ANNUAL REVIEW OF MICROBIOLOGY

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VOLUME 11

1957

ANNUAL REVIEWS, INC.
PALO ALTO, CALIFORNIA, U.S.A.

ANNUAL REVIEWS, INC.
PALO ALTO, CALIFORNIA, U.S.A.

FOREIGN AGENCY

Maruzen Company, Limited
6, Tori-Nichome Nihonbashi
Tokyo

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA BY
GEORGE BANTA COMPANY, INC.

PREFACE

The preface for this *Review* enables us once again to express our thanks to those who have collaborated in the authorship of this volume. Their surveys of advances in the various fields of microbiology are important contributions to a better understanding of microorganisms and their behavior. We regret that factors beyond our control have made it impossible to present reviews on Flagellated Symbionts of Wood Roaches and Termites, Parasitic Flagellates, Poliomyelitis, Antifungal Antibiotics, Ageing in Microorganisms, and Nematodes.

We wish to express, again, our appreciation of the contributions made to this *Review* by Dr. J. M. Sherman during the first ten years of its life, and to voice our sorrow in the loss of our friend, an outstanding figure in the field of microbiology.

We wish to acknowledge our gratitude and thanks to Dr. Monroe D. Eaton who, after ten years of valued service, has retired from the Editorial Committee. We are pleased to welcome Dr. Charles Evans as his successor.

It is a pleasure, as in past years, to extent our appreciation to the office staff of Annual Reviews, Inc., acknowledging in particular, the aid given us by Miss Beryl Daniel as Editorial Assistant.

S.P.C. T.M.S
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FINER MORPHOLOGY OF MICROORGANISMS[1]

By E. Fauré-Fremiet[2]

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The reviewer has not been able to carry out in full the assignment which the editors of the "*Annual Reviews*" kindly entrusted to him; he requests permission to limit the present paper to several general remarks based upon a few particular findings.

As is well known, our knowledge of the cell has benefited by a combination of diverse techniques which have permitted experimentation on living cellular material, microchemical or biochemical determination of its constituent parts, and analysis of its structures by refined optical methods. But it is particularly to the electron microscope that we owe revelation of a level of organization which is interposed between the fine structure visible with light microscopy and the macromolecular structure indirectly known, by colloidal properties of the cytoplasm when amorphous or by optic anisotropy and, possibly, by x-ray patterns when paracrystalline.

The philosophical and biological interest in such new aspects of cellular organization is evident, and their study indicates the rapid progress which can take place in a field which encompasses the comparative cytology, normal and pathological, of higher organisms (64). Concerning the invertebrates in general and the protists in particular our knowledge is less advanced, although much information has already been accumulated. The difficulty is that, in the case of the flagellates and ciliates, for example, intracellular differentiation often shows such extensive variety and such high complexity that interpretation of fine structures here requires numerous elements for comparison. At the present time, such elements for comparison often are lacking or are too rare and too scattered to furnish the material necessary for critical examination. But knowing the activity with which research along these lines is now being pursued in a number of laboratories, we may believe that such a situation will be modified rapidly and that certain problems posed by ultrafine structures which seem unresolvable at the present will be open to discussion, at least, in the near future. Several examples of this provisional situation will be presented in the remainder of this brief paper which, however, must not be considered as a complete discussion of the subject either bibliographically or critically.

Numerous questions are raised by the fine structure of protists. Some are related to special aspects of several problems of *general* cytology such as the structure of mitochondria, nature of the ergastoplasm, structure of nuclear constituents, etc. Others are related to problems of *comparative* cytology which must be resolved in terms of general cytology. We shall encounter

[1] The literature pertaining to this review was concluded in January, 1957.

[2] I wish to thank my colleague and friend, Dr. J. O. Corliss, University of Illinois, for translation of the text.

these two sorts of questions with regard to the chondriome and to the ciliary apparatus and its differentiation.

The Chondriome (Mitochondria)

The structural complexity of mitochondria as revealed by the electron microscope is closely comparable in the protozoa and protophyta investigated by different workers with that already known in the cells of higher organisms; there is only this difference: the internal surfaces are developed in the former (58, 59, 63) in the form of villi (microvilli) and not in the form of ridges (cristae) as in the latter. It remains to be known if this peculiarity in mitochondrial structure is uniquely and exclusively a characteristic of the protists.

Several investigations (12, 13, 51, 52) carried out with *Paramecium*, however, indicate that the electron microscopical images of mitochondrial structure as generally recognized may involve artifacts caused by certain factors in the techniques employed. On the other hand, the nature, the origin, and the differentiation of mitochondria are still problems which remain subject to much question if, with several authors, one accepts for *Paramecium* a certain "isomorphism" of mitochondria and nucleoli (12, 13, 51, 52) and for *Stentor coeruleus*, as for *Blepharisma undulans*, the identity of pigment granules with mitochondria (66).

It is with considerable reservation that the writer takes note of these diverse interpretations; his own observations with electron microscopy (18, 26, 70) have enabled him to compare the different pictures furnished by the pigmented granules of *Stentor coeruleus* and *Stentor niger*, the so-called "protrichocysts" of *Stentor polymorphus* and *Stentor roeseli*, the calcic vesicules of *Loxocephalus*, etc. Perhaps the ultrastructure of these different bodies shows some common characteristics: it appears to differ distinctly from the mitochondrial picture, but the hypothesis that the bodies could be derived from mitochondria, by an irreversible transformation, is as yet neither excluded nor demonstrated. Keeping in mind that interpretation of nuclear ultrastructure, in the ciliates, often gives rise to confusion, we must admit that more investigations, of a more precise nature, must be awaited (only a short time, perhaps) before the questions which have just been mentioned can be discussed further to any advantage.

The Ciliary Apparatus

It is now known that all vibratile cilia and flagella exhibit the same type of structure, characterized by a fibrillar bundle composed of 9 possibly double peripheral fibrils and 2 central fibrils; the bundle is embedded in an amorphous matrix itself enveloped by a fine pellicle in continuity with the cellular membrane.

Leaving to one side the case of the bacteria (1, 65), the cilia and flagella of all the protists examined by electron microscopy respond to the same structural type, the "9+2 pattern" (3, 4, 27, 38, 42, 47, 53), which may be

considered a universal characteristic, although its significance remains undetermined (1).

But the cilum, properly speaking is, like the flagellum, a caducous element and the only characteristic of permanency belongs to the basal corpuscle or kinetosome. In all the protists examined to date (17, 18, 19, 53, 62, 70) the kinetosome appears as a tubular structure; the 9 peripheral fibrils of the vibratile organite extend into its walls but the double central fibril terminates in a small thickening or apical knob. The kinetosomal tube bears median or basal thickenings associated with different accessory structures. In accord with the Henneguy-von Lenhossek theory, generalized by Chatton (9, 40), the kinetosomes, in several of their characteristics, call to mind the centrioles recently observed with the electron microscope in higher animals.

The power of kinetosomes to multiply appears to be well established by observations made at the level of the light microscope. Yet electron micrographs have not yet shown a clear-cut picture of kinetosomal bipartition; perhaps this has been just a matter of chance. Certain views, however, have suggested the possibility of a budding of the basal granule (21, 70). The choice of appropriate material for study will be necessary for full resolution of this perplexing question, the mode of duplication of the allegedly all-important kinetosome.

The ciliates present a number of structures temporarily characterized by the presence of resting kinetosomes, that is to say, noncilia-bearing kinetosomes. Several electron micrographs show these as ovoid vesicules (70); a systematic study will be needed to determine with precision this particular kind of structure and its modification during elaboration of the cilium.

The morphogenetic power of kinetosomes expresses itself in diverse ways, involving all or part of the vibratile organites themselves. Cirri and membranelles (17, 18, 19) are formed by the coalescence of adhacent cilia which adhere to one another either through means of an intermediate substance or through involvement of fine lateral expansions of the ciliary membrane. The latter situation has been observed not only in the hypotrich *Euplotes* (53) but even in *Frontonia* and several other holotrich ciliates (70); the question whether these expansions are comparable with the mastigonemes or Flimmer of flagellated protozoa (5, 6, 48, 49, 50) has not been resolved.

The cilium can become differentiated by loss of its mobility: the dorsal bristles of *Euplotes*, presumably sensory in function, serve as an example of this. The fundamental structure of a cilium is found here, but the kinetosome is located at the bottom of an ectoplasmic crypt (29, 30, 31, 70) to which are attached rod-shaped bodies of an unknown significance (9, 29, 30).

The stalk or peduncle of the peritrich ciliates is composed of a bundle of scleroprotein fibers arising at the level of a specialized ciliary region, the scopula. In several forms, e.g., *Opercularia* and *Zoothamnium*, the nine peripheral ciliary fibrils are continually lengthened at the same time that they are transformed to show a periodic structure closely resembling that of collagen fibrils. In *Campanella*, on the other hand, it is the ciliary membrane

which is lengthened in a network of fine fibrils. But, in all cases, the peduncular scleroprotein fibers result from transformation of basic ciliary constituents (54, 55).

Another differentiation of kinetosomal origin (62, 70) is represented by the trichocysts of *Paramecium* and *Frontonia*. The ultrastructure of these, now well known (2, 37, 38, 62), seems bound to tubular organites of the kinetosome type.

The base of the kinetosome is the place of origin of ciliary rootlets, fibrillar bundles appearing as various structures (18, 42, 45, 46, 53, 62), which penetrate more or less deeply into the cytoplasm. In several peritrich ciliates these rootlets fuse laterally to form a fiber showing a reticulated ultrastructure quite unique (34, 39, 60, 61, 70).

Some other structural elements also seem derived from kinetosomal bodies, for example: the uniting fiber or kinetodesma; ectoplasmic myonemes, curiously characterized by their complex leaf-like structure (18 to 22); and parabasal bodies. But the last-mentioned term, which actually has only a topographic connotation, certainly has been used to designate a number of different protistan organites. In the case of the hypermastigote flagellates, electron micrographs permit comparison of their parabasal body with Golgi apparatus (11, 33, 36), since it possesses the characteristic structure of the latter (35). This fact also has been verified for several phytoflagellates: *Chromulina* (16, 70) and perhaps *Synura* (43). On the contrary, the stigma of the phytoflagellates, which has been compared with a parabasal (32), forms an integral part of a chromoplast (7, 8, 15, 16, 28, 41, 67, 68, 69); but a flagellum happens to join it at its surface in *Chromulina psammobia*, as in the case of the spermatozooid of *Fucus* (44, 70).

Then again the parabasal bodies in rosettes, joined to the cirri and the dorsal bristles of *Euplotes* (9, 21, 30), show a granular structure very different from that of the Golgi apparatus and more nearly resemble certain intracellular glandules observed in quite different situations and roles, for example, in *Dysteria* and the chonotrichs (24, 70).

A great deal of checking and experimenting will be necessary before we can define and discuss with confidence the essential characteristics of the diverse structures mentioned above, and this is also true if one contemplates comparative study, in the protists, of the pattern of buccal organization (10), of skeletal structures (14, 20, 21, 57), of cuticular, secretory, or excretory structures (70), or of specialized organites as, for example, the suctorial or prehensile tentacles of the Suctorida (56, 59).

It may be concluded that, in opening to observation a wide domain of ultrastructures, the electron microscope necessitates revision of a number of interpretations and hypotheses concerning cellular organization. When this organization becomes complicated and diversified by a multiplicity of cytoplasmic and nuclear differentiations, as is observable in certain protists, such a revision appears particularly critical. Proper identification of what is revealed requires a great deal of discretion when elements for comparison

remain rare or scattered. But the rhythm of research in progress allows anticipation of early acquisition of sufficient data to justify hypothetical interpretations and to provide for worthwhile discussion of them.

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COLICINS[1]

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Introduction

Reciprocal antibiotic actions have often been reported in *Escherichia coli*, and are indeed very frequent in the Enterobacteriaceae. Intervention of diffusible antibiotic substances was first demonstrated in some isolated instances (72, 76, 117) but is in fact quite common (7, 28, 68, 75, 78, 82, 100, 106, 107, 112). The active strains do not all act in the same way, and they produce, according to the case, one or more different antibiotic substances, known as colicins. They, in turn, may be inhibited by strains producing other colicins (22, 28).

The name "colicin" was originally intended to characterize antibiotic substances produced by *E. coli* (22). A great many *Shigella* (19, 24), some *Escherichia freundii* (58) and fewer *Salmonella* (37, 84) also produce antibiotic substances most of which are identical with or related to those produced by *E. coli*, and must therefore be included in the colicin group. Colicins may be defined as antibiotic substances, or complexes of antibiotic substances, which are highly specific, are produced by certain strains of intestinal bacteria, and act upon other related strains.

Methods

The first requirement for demonstrating the production of colicin is a sensitive indicator strain, e.g., *E. coli* ϕ of Gratia (72). But other strains, such as *E. coli* B,C, or K-12 are equally suitable, or even any *Shigella sonnei* strain (28, 81), as they are generally sensitive to a great many colicins.

General techniques for demonstrating antibiotic action may be applied, e.g., inoculation by perpendicular streaks of active and sensitive strains, or stabbing active strains on a plate seeded with the sensitive indicator strain. But as colicins diffuse slowly in agar, the zones obtained by the simultaneous inoculation of active and sensitive strains are generally not clearly defined and are difficult to distinguish from lysogenic actions or other less specific antibiotic manifestations, such as direct antagonism, staling of media, etc., It is therefore preferable to allow the active strains to develop for 48 hr. before seeding the sensitive indicator strain. In this way much larger inhibition zones are obtained, the morphology of which is characteristic of each colicin, and in which there are often resistant colonies which are easy to pick.

Demonstration of colicinogenic properties (28).— Prospective colicinogenic strains are stabbed on ordinary agar plates (generally eight per plate) and

[1] The survey of literature pertaining to this review was completed in December, 1956.

incubated for 48 hr. at 37°C. The macrocolonies which develop are sterilized by chloroform vapor. The sensitive indicator strain is then seeded over the entire surface, either by pouring onto it 4 to 5 ml. of melted agar containing 0.1 ml. of a broth culture of this strain, or by applying for 5 min. a disc of filter paper soaked with the culture. The confluent culture developed by the sensitive strain after 24 hr. incubation reveals inhibition zones surrounding the colicinogenic colonies.

Demonstration of sensitivity to colicins (28).—When investigating the sensitivity of a single strain towards several colicinogenic strains, the above mentioned technique may be used, in which the strain being tested for sensitivity plays the part of the sensitive indicator. If the sensitivity of several strains towards a single colicin is investigated, it is simpler to make a streak of the colicinogenic strain on an agar plate and, 48 hr. later, to make perpendicular streaks of the strains to be tested. The technique of perpendicular streaks naturally is only applicable when dealing with very active colicinogenic strains.

Selective technique to isolate colicinogenic strains from feces (74).—Broth is inoculated with a sample of feces and kept at 37°C. The next day, 1 ml. of a suitable dilution (generally 10^{-5} to 10^{-7}) of this culture is seeded onto a plate of ordinary agar. Immediately afterwards, a second layer of 10 ml. of agar is poured over the first, and the plate is then incubated for 48 hr. The seeded culture develops in well isolated colonies imprisoned between the two layers of agar. Then the sterile surface of the upper layer is seeded evenly with an indicator strain. After 24 hr. at 37°C., this indicator strain develops uniformly, except in some circular inhibition zones centered in the depth by active colonies, which can easily be picked. A disadvantage of this technique is that cells from the depth are sometimes drawn to the surface when the second layer is poured. This may be remedied by chloroform sterilization immediately after the second layer has solidified. The chloroform vapors do not penetrate and only affect contaminants of the superficial layer.

Preparation of colicins in liquid medium (31).—Colicins are released in cultures of productive strains, in varying concentrations according to the strains and the type of colicin produced. Generally, colicins E and K have the highest concentrations. The sterilization of these cultures gives rise to certain problems. Colicins are very adsorbable, and are thus retained by the usual bacteriological filters (Chamberland, Berkefeld, Seitz, and Iena). Filter membranes are more suitable. The best technique consists of sterilizing with chloroform. Chloroform, 1 to 2 percent, is added to a 48-hr. broth culture, which is then shaken vigorously for 5 to 10 sec. The culture is then centrifuged and the clear supernatant contains the colicin.

Titration of colicins (52, 71, 96).— The titration of colicins is accomplished by spotting drops of a series of successive dilutions of the preparation to be titrated on the surface of a plate seeded with an indicator strain. In this way, a series of decreasing inhibition zones is obtained, ranging from complete inhibition through more and more partial inhibition to normal growth,

and forming a regular gradient over a range of dilution of approximately 1 to 100. The indicator plates are prepared by pouring 4 ml. of 7 per cent nutrient agar, to which is added 0.1 ml. of a growing broth culture of the sensitive strain, on a very even basal layer of nutrient agar.

Colicinogenic Strains

Colicinogenic strains may produce various colicins and the same strain often releases two or three distinct colicins (12, 22). During preliminary research carried out on a limited number of colicinogenic strains, 17 different colicins were demonstrated (28). If more strains are investigated, this number is likely to increase considerably, and the problem needs to be simplified. At present, colicins are grouped solely according to the specificity of their action, without taking their other characteristics into account (30). The colicins of each of these groups may be substances which are very different chemically, but they have one thing in common, namely, that they fix themselves on the same receptor of the sensitive strain. In order to determine the colicin produced by an unknown strain, its specificity must be compared with that of standard strains of each group. The best method is to obtain a mutant which is resistant to this unknown strain, and to determine towards which standard strain this resistance is developed. This determination, however, is not always easy, owing to cross-resistances, which are especially frequent between the groups B, I, and V. It is also particularly complicated when the tested strain produces several distinct colicins (47).

Colicinogenic strains do not differ from noncolicinogenic strains by any other property, but there is some relationship between biochemical and antigenic properties of the strains and the type of colicin they produce (21, 28). *E. coli* strains may produce all the types but *Shigella* and paracolon strains produce mostly type E, and *E. freundii* type A. The relationship is particularly well-defined in the case of *Salmonella*, in which the only three colicinogenic strains known, two of *S. typhimurium* (37) and one of *S. schottmuelleri* (84), are of very different origins, but all produce colicin I.

Colicinogenic properties may be used as specific markers in epidemiological studies of *Shigella* and *E. coli* in infantile gastroenteritis, colicinogenic strains being of frequent occurrence (1, 63). The colicin-typing is independent of the phage-typing, and allows a subdivision of the types. The production of colicins by some intestinal pathogens is certainly an important factor in their struggle to implant themselves in the intestine, and must therefore be considered as a factor of pathogenicity.

A relationship also seems to exist between colicinogenic strains and intestinal infections. Strains producing colicins active against *Shigella* are more frequent in dysenteric patients than in normal persons (79, 80, 105). Strains producing colicin B, one of the rare colicins active against *S. schottmuelleri*, are very frequent in paratyphoid B patients (34, 47, 67). The urine of typhoid patients regularly and consistently contains a special type of *E. freundii* producing colicin A (58).

Nature of Colicins

Colicins comprise a group of quite varied antibiotic substances, which differ in numerous characteristics (28, 46):

(*a*) Extent and specificity of the activity spectrum: The activity spectra of the different colicins are quite diverse, but always are limited to strains of Enterobacteriaceae. *Escherichia* and *Shigella* strains are often sensitive to several colicins, occasionally to them all. The action of colicinogenic strains on bacteria which do not belong to the Enterobacteriaceae family, described by some authors (14), is probably due to factors other than colicins.

(*b*) Specificity of resistant mutants: Strains sensitive to several colicins may yield mutants which are resistant specifically to a given colicin, but remain sensitive to other colicins.

(*c*) Morphology of the inhibition zones in agar: The intervention of varied colicins is expressed by considerable differences in the expanse and aspect of the inhibition zones produced by colicinogenic strains. Certain zones may reach a diameter of 50 mm., whereas others are hardly apparent. They may be without growth, reveal a continuous partial growth, or a variable number of resistant colonies. Their edges may be clear-cut or gradual, sometimes rosette-shaped, and surrounded later with a zone of secondary lysis.

(*d*) Diffusibility in agar: Colicins diffuse relatively slowly in agar, at a variable rate largely determining the dimension of the inhibition zones. The most diffusible colicins pass through cellophane, but others are kept back.

(*e*) Thermoresistance: Certain colicins withstand heating at 100°C., while others are altered or even destroyed at 60 to 70°C.

(*f*) Sensitivity to proteolytic enzymes: Colicins are destroyed more or less rapidly by proteolytic enzymes. Colicin V is the most sensitive and colicin C the most resistant.

(*g*) Lastly, they differ according to antigenic properties (8, 71, 85) and electrophoretic motility (101).

The diversity of characteristics of the different colicins points to very different chemical constitutions. Purification experiments on colicin V and two others gave products having the general reactions of polypeptides or proteins (16, 69, 83, 88).

Colicin K was recently purified by repeated precipitations with ethanol and ammonium sulphate, followed by extraction with chloroform-octyl alcohol (70). The product obtained is a substance soluble in water, colourless and free from nucleic acid. It contains 6.5 per cent nitrogen and 1.6 per cent phosphorus. It displays only one electrophoretic component, but seems to be heterodisperse on ultracentrifugation. It appears to be a macromolecular substance, consisting of carbohydrate, protein, and lipide. It has a powerful antibiotic action, for one drop of a solution containing 1 μg/ml. totally inhibits the growth of the sensitive strain. The chemical, physical, immunological, and toxic properties of this product point to the somatic O antigen of the bacteria, but it is not excluded that colicin K may, in reality, be a distinct molecule linked to this lipocarbohydrate-protein complex (70). The

study of inactivation curves of colicin K by x-rays leads to the conclusion that it has a molecular weight somewhere between 60,000 and 90,000 (96).

Mode of Action of Colicins

Colicins act on sensitive strains through the agency of specific receptors (23). Indeed, a strain which is sensitive to several different colicins does not present a single point of attack, characteristic of its type of sensitivity and identical for all colicins to which it is sensitive, but has several receptors, specific for each of these colicins. A resistant mutant of a strain sensitive to several colicins does not lose in one piece the general sensitivity of this strain, but only the sensitivity to the particular colicin which selected it, and maintains at the same time, towards other colicins, a sensitivity identical to that of the strain from which it was derived. By a series of successive mutations, it is possible to transform a strain, which was originally sensitive to numerous colicins, into a completely resistant strain by causing it to lose successively all the receptors which characterize it (28) (Fig. 1).

These colicin receptors are in fact fixation-receptors (10, 42). Extracts of sensitive bacteria specifically neutralize colicins by fixing them. Similar extracts prepared from resistant bacteria have no action (10, 11). The presence of fixation-receptors is also proved by the action of antibacterial serums. These serums do not have any direct anticolicin action, but protect sensitive bacteria against the later action of colicins, probably by blocking the receptors (9).

Fixation on a specific receptor is an obligatory requirement for the action of a colicin. It is, however, insufficient and certain strains may fix a given colicin and yet be insensitive to its action. When sensitive cells adsorb colicin, they are rapidly killed. Colicins are bactericidal, but not bacteriolytic, agents.

The kinetics of the bactericidal action have been studied with regard to colicins ML, E and K (42, 64, 91). The proportion of surviving cells is in direct relation with the number of cells upon which the colicin was acting. It is, on the other hand, an exponential function of the time of action, and of the concentration of the colicins. The survival curves, therefore, reveal the adsorption phenomenon which is the basis of the action of colicins.

The bactericidal action of colicins is accompanied by profound modifications in the metabolism of the sensitive bacteria. After addition of colicin ML to a culture of these bacteria, growth immediately comes to a stop. Oxygen uptake remains at its initial rate for about 20 min., then gradually decreases. The ribonucleic acid and deoxyribonucleic acid syntheses are immediately stopped (91). Colicin may block all bacterial syntheses. It also blocks the reproduction of certain bacteriophages (48, 91).

Relations between Colicins and Bacteriophages

Gratia (73) had already been struck with the resemblance which exists between the coli V-coli ϕ antagonism and the phenomenon of bacteriophagy, and many authors studying colicinogenic strains have first thought of them as lysogenic strains. This resemblance is not confined to mere anal-

ogies and leads to the conclusion of some relationship between colicins and bacteriophages (18, 35, 46).

Lysogenic and colicinogenic strains.—Lysogenic strains, like colicinogenic strains, are very frequent in the Enterobacteriaceae, and have a similar geographic and animal distribution. By the use of selective techniques, it is possible to reveal the presence of colicinogenic strains in practically all human and animal stools studied (74). They appear in the first days of life and are found in individuals of all age.

Colicinogenic properties, like lysogenic properties, are extremely stable hereditary characteristics. The V coli strain, isolated by Gratia in 1925, still produces the same colicin 30 years later. Strains lyophilized and preserved for over 15 years produce colicin in the first broth passage (108). Colicinogenic strains may occasionally vary and give rise to noncolicinogenic mutants, just as lysogenic strains may give nonlysogenic mutants. Lysogenic strains often release several different phages. Colicinogenic strains may also produce several distinct colicins (28).

Lysogenic strains are never sensitive to the particular phage they carry but may be sensitive to other bacteriophages. This immunity is a direct consequence of the lysogenic property, for nonlysogenic mutants are generally sensitive to the phage of the mother strain. Colicinogenic strains behave in the same manner. They may be sensitive to other colicins, but never to the particular colicin they produce. If they lose their colicinogenic property by mutation, they become sensitive to the colicin they originally produced (28).

The production of colicin by colicinogenic strains is strictly parallel to the liberation of phage by lysogenic strains. In a lysogenic culture, the spontaneous production of phage particles results from lysis of only a few rare cells; but massive lysis, with production of phage particles by almost all the cells, may, however, be induced by various mutagenous or cancerigenous agents, in particular ultraviolet irradiation (104). Jacob, Siminovitch and Wollman (90, 91, 103) discovered that the colicinogenic strain *E. coli* ML, when induced, lyses and releases colicin, just as a lysogenic strain, when induced, lyses and releases phage. The cytological aspect of this lysis has been described by Delaporte (15). In fact, this ML strain is not only colicinogenic but also lysogenic, and lysis is a result of the induction of its prophage (52, 95). But colicinogenic strains, which are certainly not lysogenic may also be induced to produce colicin, without, however, lysing (52, 54, 87, 95, 109). Colicins are agents which kill but do not lyse. Colicinogenic bacteria, like lysogenic bacteria, perpetuate by heredity a potential lethal factor, whose development may be induced, in both cases, by the same agents.

Induced lysogenic or colicinogenic bacteria undergo fairly comparable metabolic modifications (92). After induction, residual growth with parallel augmentation of respiratory intensity can be observed in both cases. The syntheses continue at a reduced rate, except for that of deoxyribonucleic acid,

which is temporarily blocked in lysogenic induction, but not in colicinogenic induction. This difference is probably due to the fact that bacteriophages contain a deoxyribonucleic fraction, whereas colicins are basically of protein nature.

Colicinogenic strains, therefore, behave in many respects like lysogenic strains. These two properties are, however, completely independent and distinct, although they may be found together in the same strain (5, 33).

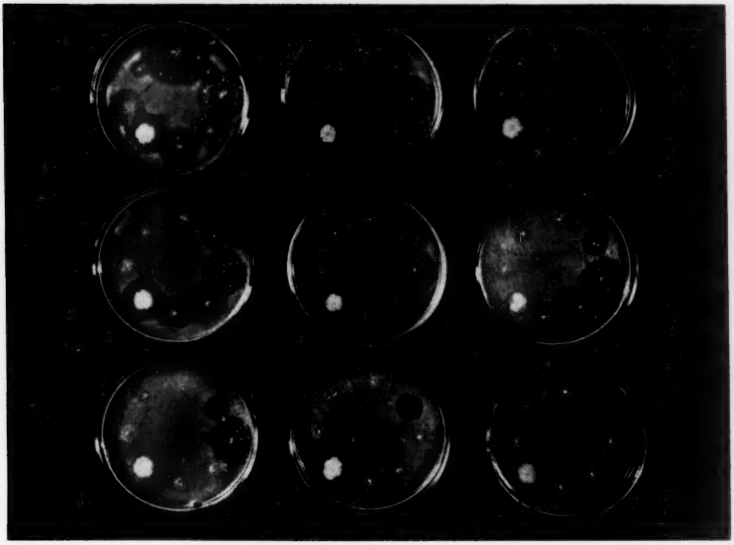

FIG. 1. Successive loss of colicin receptors by mutations in series in a strain of *Shigella sonnei*. The 9 plates were inoculated with the same series of 8 different colicinogenic strains. Starting at the top left-hand corner, the sensitive cultures are: a mother strain of *S. sonnei* susceptible to the 8 colicins; a mutant derived from it by resistance to strain No. 1; a second mutant derived from the first by resistance to strain No. 2. Successive mutants were derived each time from the preceding one by resistance to a new strain until complete resistance was obtained.

Colicins and bacteriophages.—Just as colicinogenic strains resemble lysogenic strains, so colicins resemble bacteriophages, but more especially virulent bacteriophages. These two agents have activity spectra which are very comparable in their diversity. In both cases, specificity is extremely pronounced, and depends on each colicin or bacteriophage being considered (28).

The varying appearances of the inhibition zones induced in agar by colicins reproduce, in fact, to a much bigger scale, every morphological modality

of the plaques induced by bacteriophages. Specificity of the inhibiting agent and nature of the inhibited strain intervene in both cases (46).

Sensitivity to various colicins is determined by a series of receptors which are specific for each colicin (28). These colicin receptors behave exactly like bacteriophage receptors. They fix and neutralize the corresponding colicin and they may be blocked by the addition of an antibacterial serum (9, 10). The presence of colicin receptors, like that of bacteriophage receptors, depends on genetic factors susceptible to recombination by crossing (59, 94) or to loss by mutation. Mutations affecting these receptors are specific, and occur spontaneously and independently of the other characteristics of the strains being studied (26, 27)

Colicin receptors behave exactly like bacteriophage receptors, but are generally independent and separate from them. Systematic investigation has, however, shown that a specific reciprocal cross-resistance occurs constantly in the case of certain colicins and definite bacteriophages, and leads to the conclusion of single receptors common in some cases to a colicin and a corresponding bacteriophage (65). Thus, all resistant mutants, selected by both colicin K and bacteriophage T6, always resist these two agents simultaneously (66). This cross-resistance behaves as a single marker in genetic crossings (59).

The lethal action of colicins is comparable to that of virulent bacteriophages. As soon as they are fixed on sensitive bacteria, growth and synthetic activities are immediately blocked (92). The addition of colicin to bacteria infected with a virulent bacteriophage also stops the development of this phage, but there sometimes arise phenomena of interference and mutual exclusion between colicins and bacteriophages, comparable to those observed between phages (48).

Whereas colicins completely suppress all syntheses, virulent bacteriophages, however, do not definitely block the syntheses which serve their own reproduction. This particularity results from the fundamentally different nature of colicins and bacteriophages. Colicins are inert chemical substances which kill the cells without being reproduced, whereas bacteriophages are biological entities, which are reproduced and mutliplied by the cells they kill.

Bactericidal action of bacteriophages.—Colicins differ from bacteriophages by the fact that they do not multiply and their action is not transmissible in series (32). Certain bacteriophages (36, 38, 39, 77, 114), and in particular bacteriophage T6 (40), of which we just mentioned the relation with colicin K, have upon certain strains, a bactericidal effect which is not accompanied by multiplication of the particles, and is consequently not transmissible in series. In these conditions, the action of these phages strongly resembles that of colicins, and never occurs in the form of isolated plaques, characteristic of phage multiplication. This bactericidal action does not depend on an antibiotic substance which may be present in the phage preparations, but is linked with the particles. In fact, it disappears after neutralization by antiphage serum or fixation on sensitive bacteria (77), and study of the kinetics reveals

that the proportion of surviving cells is always equal to the proportion of cells which have not fixed particles, calculated according to Poisson's formula $P(0)=e^{-n}$, when n is the average number of particles fixed per cell (44).

The dissociation of a bactericidal action from the ability to multiply has also been observed by causing certain phages to act in the absence of growth factors (110), in the absence of calcium (2, 4), in the presence of proflavine (20), or at high temperature (6). It has also been provoked artificially by adding phages "inactivated" by x-rays (115), ultraviolet rays (13, 17, 102, 111), osmotic shocks (89), or photodynamic action (116) to bacterial strains which normally ensure their multiplication. The bactericidal action of these phages is an independent property, which is quite distinct from the ability to multiply (43). The constituent of the phage responsible for this action seems to be a protein localized in the extremity of the tail of the particle (3, 89).

The lethal proteins of certain phages have close relationships with certain colicins, for example the lethal protein of phage T6 and colicin K. Indeed, these two agents have an identical specificity of action and fix themselves on the same receptor of the sensitive bacteria (41). Moreover, their inactivation-curves by x-rays are completely superimposable, and indicate an identical radiosensitive volume (96). The lethal protein of T6 and colicin K are, however, serologically distinct (45, 70), just as T6 is distinct from other phages with the same specificity of action (45).

Despite the analogies, even despite the fixation on common receptors and the intervention of substances related to colicin in the constitution of certain bacteriophages, colicins and bacteriophages are agents of abasically distinct nature. Colicins are inert chemical substances, proteins. Bacteriophages are much more complex particles, which include a deoxyribonucleic core and a protein envelope. Bacteriophages are biological units which are transmissible, reproduced and multiplied during lysis, and endowed with genetic continuity. They thus are related to viruses and genes.

The property of producing a colicin seems to be a normal, hereditary physiological characteristic of the bacteria, and must therefore be determined by a specific gene. In that case, there arises the question as to whether the relationship between colicins and bacteriophages is not, in reality, a relationship of phages, not with colicins, but with the genes which determine their production.

Genetic Factors Determining Colicinogenesis

The genetic factors governing colicin production were studied in transduction and recombination experiments (57).

Transduction of colicinogenic properties.—Certain strains can transmit their colicinogenic property to noncolicinogenic strains by culture in common. The efficiency of this transfer is quite variable according to the cases, and a selective technique is often required to demonstrate it (49). A colicinogenic strain, sensitive to streptomycin, and a noncolicinogenic strain, re-

sistant to this antibiotic, are inoculated in the same broth. After 24 hr, growth, successive dilutions of this mixed culture are spread onto the surface of agar plates containing streptomycin; then a second layer of agar with streptomycin is poured on top. The colonies which develop between the two layers of agar all come from the noncolicinogenic strain, as the other is inhibited by streptomycin. Those of the colonies which have become colicinogenic are then located, by seeding the whole surface of the upper layer, 48 hours later, with an indicator strain, sensitive to colicins (and obviously resistant to streptomycin). The next day, the confluent culture of this indicator strain reveals circular inhibition zones centered in the depth by colicinogenic colonies which are easily picked (Fig. 2).

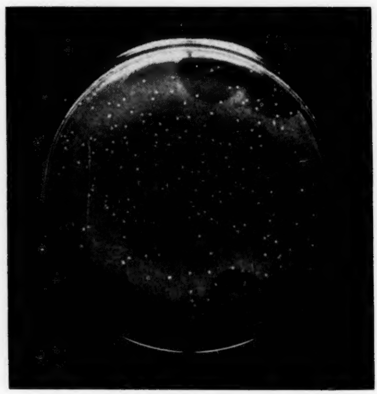

FIG. 2. Selective technique showing transduction of the colicinogenic property.

It was possible by this technique to transmit specifically to a strain of noncolicinogenic *E. coli* the power of producing different colicins, by using, as the initial colicinogenic strain, not only *E. coli* but also *S. sonnei*. The transformed cells of *E. coli* keep all the properties which characterized the initial noncolicinogenic strain, and differ from it only by the newly acquired colicinogenic property. For example, a strain of *E. coli*, transformed by mixed culture with a colicinogenic *S. sonnei*, retains not only the features of sensitivity or resistance to phages and antibiotics which mark the original strain, but also all the properties which fundamentally distinguish the *E. coli* and *S. sonnei* species, among others motility, production of gas by fermentation of glucose, fermentation of lactose and xylose, and production of indol. In the same way, it was possible to transmit, without modifying their other characteristics, the colicinogenic properties of *E. coli* to other species, *S. sonnei* (49), paracoli Ballerup, paracoli intermedium, *Klebsiella pneumoniae, Salmonella typhosa and Salmonella schottmuelleri* (86).

This isolated genetic transfer of a single trait in a mixed culture of two strains comes within the range of phenomena which Lederberg (98) called transduction. This term is often used in the restrictive sense of a genetic transfer by means of phage particles, but phages play no part in the transduction of colicinogenic properties (50). By use of adequate selective techniques, it was possible to demonstrate transduction in series, a strain already transduced being able, in its turn, to transduce another. Transduction in series, investigated in the case of *E. coli* K-12, revealed that transducing activity is linked with the sexual polarity F+. An F− strain cannot transmit its colicinogenic feature unless it is first transformed into F+ (51).

Transduction is specific for each colicin considered. For example, noncolicinogenic strain K-12, transduced by strain CA-18, produces colicin B like CA-18. The same K-12 strain, transduced by K-235, produces colicin K like K-235, and so on (49, 55). A strain, which has already been transduced for a given colicin, may again be transduced by a strain producing another colicin, and thus release at the same time two distinct colicins (51).

The efficiency of transduction varies a great deal according to the case. Generally speaking, the transformed cells represent one to ten per cent of the nontransformed cells, but the proportion may reach 100 per cent, when the strain to be transduced is very sensitive to the colicin of the transducing strain. In this case, the noncolicinogenic cells are killed, and the only cells to develop, together with the initial colicinogenic cells, are the transformed cells, which are henceforth immune, as we shall see later, and possibly spontaneous resistant mutants (49).

The transfer of the colicinogenic property is extremely rapid, at least its initial stage. The transfer can be obtained by simply mixing two cultures, colicinogenic and noncolicinogenic, just for a few minutes. So far, it has been impossible to extract the transducing agent or even to separate it from the living cells.

All colicinogenic strains are not necessarily capable of transducing this property, possibly on account of a sexual polarity F−. But other factors are involved, for strains producing several colicins usually transmit only one of their colicinogenic properties. The capacity of transduction seems to depend on the type of colicin produced. It can very often be observed with strains producing colicin I, less frequently with strains producing colicins B, E, or K, and quite rarely with strains producing colicin V (55).

Recombination of colicinogenic strains.—Transduction of colicinogenic properties to fertile *E. coli* strains, for example K-12 or B, made it possible to study the behaviour of the genetic factors which determine colicinogenesis in cross experiments. Most of the parents investigated were derivatives of strain K-12 transduced by strain K-30 and producing colicin E(I), also known as colicin ER.

Recombination in *E. coli* can be studied by crossing complementary auxotrophic parents and selecting prototrophic recombinants (97). The process of recombination obeys very definite laws. The frequency of transfer

of various properties is determined by their location with regard to the selected markers and by the F polarity of the parents (99). The crossing of colicinogenic strains with noncolicinogenic strains shows that colicinogenic properties behave in a completely abnormal way and are not linked to any studied marker (60). When they characterize the F− parent, they are transfered without exception to all recombinants, whatever markers may be involved in the selection. The inversion of the sexual polarity F of the parents, does not invert the frequency of transfer of these properties. Still some recombinants (up to 70 per cent) inherit colicinogenic properties when they are borne by the F+ parent (61). The crossing of two different colicinogenic parents may give recombinants producing the two colicins simultaneously (62).

Colicinogenic properties may reduce, or even completely suppress, the fertility of *E. coli* strains, to a more or less pronounced degree, according to the type of colicin produced (62). This reduction, already visible in crossings where the F− parent is colicinogenic, is particularly marked when the colicinogenic property is borne by the F+ parent. This inhibition is probably only apparent, and very likely results from the destruction of the recombinants, owing to the induction of colicin production during recombination. Induction by recombination was also demonstrated in lysogenic strains (53, 93). Despite their role in the fertility of crosses, colicinogenic properties do not seem to influence the frequency of transfer of other markers, and this confirms that they are independent of the normal genetic structure of the bacteria.

Immunity and resistance.—Strains rendered colicinogenic by transduction or recombination are no longer sensitive to the colicin they now produce. They acquire simultaneously the hereditary property of producing a colicin and immunity to this colicin. This immunity is independent and distinct from resistance through loss of receptor. It is quite comparable to that of lysogenic strains to the phage they carry (56). For example, strains sensitive to the colicins of group E have a common receptor. Resistant mutants lose this common receptor and resist simultaneously all the colicins of the group and phage BF-23, which adsorbs on this same receptor (29). On the other hand, strains rendered colicinogenic for a colicin of this group are solely immune to that particular colicin, but remain sensitive to the other colicins of the group and to phage BF-23.

This immunity is more or less marked according to the type of colicin produced, and is, however, never complete. Transduced strains for colicin V, in particular, remain partially sensitive to the colicin they produce. Broth cultures of these strains are less turbid than those of the same strains before transduction; agar streaks develop poorly and isolated colonies are small and irregular (56). A strain spontaneously colicinogenic and sensitive to the colicin it produces has, moreover, already been described (113).

The genetic factors which govern colicinogenic properties behave as

pathogenic agents. Transduction introduces into transduced cells a potential lethal factor, the spontaneous or induced development of which results in the death of these cells. As these genetic factors are pathogenic agents, which are transmissible and independent of the normal genetic structure of the bacteria, they might be considered as bacterial viruses, distinct from bacteriophages. Their relations with the latter point to a possible common parental ancestry.

Conclusions

Numerous strains in the family Enterobacteriaceae produce various antibiotic substances known as colicins. These colicins have activity spectra which are very diverse, but strictly limited to other strains of the same family. They are protein or polypeptide substances of high molecular weight which kill sensitive cells by fixing themselves on specific receptors.

Colicins exhibit striking relationships with bacteriophages. Colicinogenic strains produce colicins, just as lysogenic strains produce bacteriophages. Certain definite colicins and bacteriophages fix themselves on a common receptor of the sensitive bacteria. The mode of action of the colicins is very similar to the bactericidal action of certain virulent bacteriophages. The bactericidal constituent of these phages is a substance related to colicins.

Colicin production is governed by the presence of genetic factors independent of the normal genetic structure of the bacteria. These genetic factors behave as transmissible pathogenic agents and may be regarded as bacterial viruses distinct from bacteriophages. Their relationships with the latter indicate a possible common parental ancestry.

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VARIATIONS IN ANIMAL VIRUSES[1]

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Recent advances in the techniques of study of animal virus genetics have made possible a more precise approach to investigations of variations in animal viruses.

Virus strains are classified according to relatively stable hereditary resemblances between them, and a variant virus is one arising from the original or parent strain, and differing from it in an observable way. It is well-known that variations occur in the majority of viruses. In a long review, Findlay (1) has described many variations of different viruses, and has pointed out that it is desirable to know the capacity of different viruses for variation, as well as how the variants arise and establish themselves.

Variants have been recognized in the field and during laboratory manipulation, where they have been isolated by accident or in an especially selected environment. In 1884, Pasteur and co-workers (2) described the isolation of "fixed" rabies virus from the naturally occurring virus of the streets. By passage of the street virus serially in rabbit brain, it was possible to obtain a virus which produced a milder disease than the original strain, and was characterized by a short, constant incubation period and limited ability to spread through the nervous system. The fixed virus was less virulent for man and dog. The principle of passage of virulent strains in new hosts has been applied extensively in attempts to produce attenuated strains for use as vaccines. Striking success was achieved with the isolation of the 17 D strain of yellow fever obtained by passaging the Asibi strain in mouse and chick embryo tissue, with resultant loss of virulence for man and monkey [Theiler & Smith (3, 4)]. It was noted, however, that the process was difficult to repeat, and that certain sub-strains of 17 D had regained virulence [Fox & Penna (5)]. More examples of reconversion of "fixed" attenuated strains to the virulence of the parent strains have been reviewed by Findlay & Clarke (6). From earlier work, which was naturally concentrated on the practically important problems of variations in pathogenicity and antigenic structure, certain broad principles have emerged. Adaptation of a virus to pathogenicity for a new host may result in changed virulence for the original host, as well as changes in other properties. Virus strains from a common source handled in many laboratories may differ markedly from the original strain, and from each other. Repetition of certain manipulations which once resulted in the production of a well-defined and stable variant may not necessarily give the same result a second time. In other words, although variants have

[1] The survey of literature pertaining to this review was completed in January, 1957.

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been recognised frequently, their properties have been generally unpredictable, and the processes of obtaining them unreproducible.

Very little systematic work has been done to elucidate the processes by which variants have arisen. The possibilities exist that variations may be hereditary or nonhereditary changes. Inheritable variations may be due to mutation, or some process of transfer of genetic material from one virus strain to another. Such variants may have been present in the original virus population under investigation or have arisen during experiment. In these cases, variants would dominate the population in environments more favorable to their growth than that of the original strain. On the other hand, hereditary or nonhereditary changes may be induced in a virus population by pressure of environmental conditions. Before any conclusions can be drawn about how variants arose and became established, it must be reasonably certain that the original population arose from a single virus particle. Means must be available for separating mixtures of different strains, and of maintaining stable lines of virus for further study. These criteria are fulfilled by recently developed methods of isolating pure clones of virus by selecting single pocks or plaques, or by selecting virus populations which have grown at the limiting dilution at which infection takes place. An account of these techniques will be given with examples of how they have been applied in the study of virus variation.

In a short review it is obviously impossible to list and discuss the variations in almost every conceivable property which have been described in almost every virus so far isolated. Variations in morphology, antigenic structure, pathogenicity, virulence, and incidentally in other properties linked with these, will be discussed, with emphasis placed on those studies where genetic principles have been applied, and attempts have been made to elucidate the process by which variants have arisen. Until recently these criteria have been fulfilled largely by work done on influenza viruses. In future genetic studies, the emphasis will no doubt shift to viruses which readily produce plaques on monolayers of cells in tissue culture.

Establishment and Maintenance of Pure Lines of Virus

For many years the methods of cultivating and passaging viruses have involved the transfer of a number of organisms from one animal or tissue culture to another. It was not known whether or not the seed material was homogeneous, or how many virus particles were present in the inoculum. At present three methods are available for establishing pure lines of virus, presumably resulting from multiplication of a single virus particle: first, isolation of single pocks produced by inoculation of the chorioallantoic membrane of the developing chick embryo; secondly, subculture from plaques appearing on monolayers of tissue culture; and thirdly, the limiting dilution technique.

Since the demonstration by Woodruff & Goodpasture (7) of the ability of fowl pox virus to produce lesions on the chorioallantois, the method of chorio-

allantoic inoculation has been used extensively in the cultivation of viruses, notably those of the *Poxvirus* group. The method has been used for titration of viruses, as there exists an approximately linear relationship between the dilution of virus suspension inoculated and the number of lesions appearing on the membrane [e.g., Beveridge & Burnet (8)]. This linear relationship provides good evidence that each lesion is produced by one virus particle (9, 10, 11).

One of the most interesting questions in variation in the *Poxvirus* group is the origin of the vaccinia virus, used for vaccination against smallpox and studied extensively in the laboratory. Some vaccinia strains have been reputed to have been derived from human smallpox by passage through laboratory animals. Downie & Dumbell (12) favour the view that most of the current strains were derived from cowpox. Downie & Haddock (13) and van Tongeren (14) have isolated variants of cowpox from single pocks growing on the chorioallantoic membrane. The variant differed from the parent strain in that it produced lesions lacking a central haemorrhagic area. The variants were isolated in pure culture by repeated inoculation of the chorioallantoic membrane and were found to breed true. After 17 passages, there was no reversion to the original type. The variant pocks resembled those of vaccinia, but the virus was less virulent for mice and rabbits than vaccinia. It was concluded that the phenomenon represented a mutation occurring with unusually high frequency, for the red lesions of cowpox always gave rise to about 1/40 white lesions. The variant never overgrew the parent strain, and was selected because of its distinctive appearance.

The pock method has been utilized for separating mixtures of herpes strains, which differed in the size of the pock they produced, and to establish and maintain pure lines of virus [Wildy (9)]. Fenner and co-workers (10, 15) and Mykytowycz (16) have used the method for obtaining pure lines of different strains of myxoma virus.

In 1952 Dulbecco (17) developed a technique for producing plaques on monolayers of chick embryo fibroblasts. He found that Western Equine Encephalitis and Newcastle Disease viruses produced clear plaques. The method has since been applied to poliomyelitis (18), vaccinia (19), fowl plague (20), influenza (21, 22), foot-and-mouth disease (23), Rift Valley fever (24), herpes B, Coxsackie, and Enteric Cytopathogenic Human Orphan (ECHO) viruses (25, 26). It fulfils the stringent requirements of quantitative work with animal viruses since each plaque is produced by one virus particle, pure lines may be established, mixtures may be separated, the method is accurate, and a uniform type of host cell is used [Dulbecco (17), Dulbecco & Vogt (18)]. Variants showing different plaque sizes have been isolated from strains of poliomyelitis virus (27, 28). The changed properties of the variants persisted on further passage.

The so-called "limiting dilution technique" involves isolation of virus populations from a single egg, tissue culture, or animal inoculated with a dilution of virus beyond the 50 per cent infectivity end point. The principles

and uses of this technique with regard to animal viruses were first demonstrated by Burnet & Bull (29). On primary isolation from a human patient, influenza A virus grew only in the amniotic cavity of the developing chick, not in the allantoic cavity, and it agglutinated human or guinea pig red blood cells, but not fowl cells. This virus was said to be in the O or original phase. Passage in the allantoic cavity led to the appearance of the D or derived phase which readily agglutinated fowl cells. Other differences were noted between O and D phase viruses. O virus, presumably the human pathogen, did not react enzymically with ovomucin whereas the nonpathogenic D virus did. Further work by Burnet & Stone (30) showed that intermediate phases might also appear. Burnet and Bull showed that O phase virus could be maintained as the major component of the virus population in the amniotic cavity at limiting dilutions. Passage in the allantoic cavity at lower dilutions resulted in isolation of D virus. The authors concluded from their data that D mutants appeared and, in the allantoic cavity, rapidly overgrew the original strain, the mutation rate being of the order of $1/10^5$ per duplication with D multiplying at about twice the rate of O.

Differences in agglutinating power of newly isolated influenza A viruses and well-adapted derivatives were confirmed by Dudgeon and co-workers (31) and by Hirst (32), although Hirst was unable to maintain virus in the O phase by amniotic passage. Mogabgab *et al.* (33) were able to maintain virus in the O phase by passage in tissue culture of human embryonic lung. Magill & Sugg (34) objected to the conclusions drawn by Burnet and Bull on the genetic origin of the O→D change, on the grounds that O virus could be transformed to D by simple adjustment of the pH of the suspending fluid. Burnet *et al.* (35) showed that, whereas the intermediate phase virus could be so altered in agglutinating power, no manipulation which they tried could make true O virus demonstrably agglutinate fowl cells. Stone (36) confirmed that O and D virus reacted differently with soluble inhibitors, but could find no explanation for the pathogenicity for humans of O virus.

The work done on O—D variation was the first genetic analysis of variation in animal viruses. It demonstrated that O virus could be maintained in a neutral environment, equally favorable to both types of virus, by passage at limiting dilution. D virus appeared when a larger amount of virus was inoculated, and the environment was favorable to its multiplication but not that of the original strain. It was thus concluded that the differences between O and D were genetic in origin, D being a spontaneous mutant.

Further applications of the limiting dilution technique were demonstrated by Isaacs & Edney (37, 38). Working with an influenza A strain MEL they found that when the seed virus was inoculated at a dilution of $1/10^7$, the virus harvested behaved differently from the usual harvest of the seed inoculated at $1/10^4$. The variant differed in enzymic activity, serological reactions, and virulence for mice. By inoculating eggs with a range of dilutions, they found that harvests from all except the highest dilution behaved like MEL or *M* whereas the highest dilution yielded two sorts of virus fluids be-

having like *M* or the variant *m*. The strains isolated from the highest dilution bred true on further passage irrespective of the dilution at which they were inoculated. It was concluded that the stock seed represented a mixture of *M* and *m* viruses. When both were present *M* overgrew *m* and the resultant harvest behaved like *M*, a fact which was confirmed by inoculation of artificial mixtures of the two strains. However, at the highest dilution where it was assumed that infection by a single particle had taken place *m* only, or *M* only, grew. No explanation of the origin of *m* was offered and attempts to isolate *m* after repeated passage of a pure line of *M* failed.

Mixtures of *M* and *m* were thus separated by the limiting dilution technique, and variants were isolated by this method from stock seeds of three other influenza strains. Such separation can be expected only of the major component of a virus population, or of almost equal mixtures, in a non selective environment. Other variants were isolated from pure lines of *M* and *m* by passing the viruses in excess antiserum or in the presence of the receptor-destroying enzyme from *V. cholerae*, RDE (39). These variants *Ms* and M_R were again separated by the limiting dilution method and shown to breed true on removal of the selective environment.

A pure virus population could be maintained by passing at limiting dilution only if any variants which arose were of low frequency, since only the major component of the population would be reinoculated. On the other hand, if variants were required, a large inoculum would be more likely to yield them. This was demonstrated by Burnet (40) in experiments where repeated passage of influenza strain NWS at limiting dilution resulted in the maintenance of the original properties, whereas repeated passage at low dilution yielded a variant differing in many properties from the original strain. Sabin *et al.* (41) isolated variants of the three immunological types of human poliomyelitis virus if large inocula were used for repeated passage, but not if small inocula were used.

Discussions abound on the validity of the assumptions made in applications of the limiting dilution technique; whether infection by a single particle has taken peace, how many virus particles constitute one infective dose, and the effect that variations in host susceptibility could be expected to exert (11, 42, 43, 44). As well as in the examples mentioned above, the method has certainly been applied successfully in recombination experiments with influenza viruses (44), in separation of a variant of Rift Valley Fever virus (45), and in testing the stability of properties of variant viruses (e.g., 46, 47, 48, 49). The results justify its use until more rigid methods can be applied to all viruses.

Variations in Morphology

Mosley & Wyckoff (50), using the electron microscope, observed filamentous forms as well as the usual spherical forms in preparations of influenza viruses. Chu, Dawson & Elford (51) showed that the filaments possessed the same antigenic and haemagglutinating properties as the round

forms. Earlier work suggested that spheres were formed by the break down of filaments [Murphy, Karzon & Bang (52), Wyckoff (53)]. Donald & Isaacs (54) found that mechanical break down of filaments by ultrasonic vibration resulted in the formation of spheres and short rods, an increase in haemagglutinin titre, but no change in infectivity. The authors concluded that simple break down of filaments could not explain the formation of fully infectious spherical forms. Electron microscopic examination of ultrathin sections revealed that filaments and spheres were formed by different processes, and that previously observed segregation of filaments was probably due to artifacts [Morgan *et al.* (55)].

Quite a lot of evidence has accumulated that the ability to form filaments is an inherited character. Abundant production of filaments was characteristic of certain influenza A strains, particularly freshly-isolated strains. However, changes in morphology were observed after repeated passage in mice and eggs, and well-adapted strains tended to show only spherical forms (51). Burnet (personal communication) has found that repeated passage at low dilution results in a sharp fall in the proportion of filaments to spheres, whereas passage at limiting dilution allows indefinite maintenance of a high filament/sphere ratio. This is taken as evidence that a mutation was involved in the loss of the filament-forming character of the virus. In recombination experiments, crosses have been carried out between filamentous and nonfilamentous strains. In progeny bearing the serological character of the filamentous parent strain, acquisition of pathogenic properties of the nonfilamentous parent was accompanied by the loss of capacity to produce filaments (56).

Filamentous forms also were observed in preparations of Newcastle Disease virus, when the virus particles were suspended in hypertonic salt solution. Although full infectivity was maintained, Bang (57) concluded that these filamentous forms were artifacts in the sense that only spherical forms were observed in solutions of physiological saline. This may be considered as a variation dependent entirely on the environment.

Antigenic Variations

Antigenic variations in pathogenic viruses are of obvious importance in the choice of strains for use as vaccines. The vaccine used against influenza in the 1946–47 epidemic failed dramatically because the vaccine strains bore little antigenic resemblance to the strain which caused the epidemic [Francis, Salk & Quilligan (58)].

Antigenic differences among strains of influenza A virus isolated during different epidemics were recognized soon after the first isolation of the virus [Magill & Francis (59)]. Studies using virus-adsorbed sera have shown that a number of distinct antigens exist, and that influenza A strains fall into groups with a tendency, in succeeding epidemics, for old antigens gradually to be replaced by new ones [Friedewald (60), Hirst (61); Jensen & Francis (62)]. It has been suggested that complete loss and replacement of antigens

does not take place and, in fact, only a finite number of variations are possible [Francis (63), Jensen (64)]. If this were so, reversion to earlier types of viruses would be expected, but so far, when strains prevalent many years previously have been isolated, there has been at least a strong suspicion that they were contaminants [Andrewes *et al.* (65), Isaacs & Andrewes (66)]. Hirst (67, 68) thought that passage in animal hosts before serological testing was responsible for some of the antigenic differences, but later he showed changes brought about by this method to be minor in nature (61). Earlier it was thought that only one antigenic variety of virus would be isolated from one epidemic (59, 67), but later it was demonstrated that often more than one could be isolated (e.g., 46, 61, 69). The time of dominance of one antigenic type in epidemics has been shown to range from two to nine years (61). Influenza B viruses, although not showing as great a variability as influenza A (70) have shown variation in antigenic pattern over the years (71). The influenza C viruses so far isolated have all been antigenically similar (72).

Another type of antigenic variation in influenza viruses, P—Q—R variation, has been described by van der Veen & Mulder (73) and by Isaacs (74). P virus is inhibited to a high titre only by homologous antiserum, Q virus to a low titre even by homologous antiserum and R virus to high titres by antisera prepared against all three variants P, Q, and R. Naturally occurring P and Q viruses have been isolated at limiting dilution, and bred true on further passage in eggs, which suggested that P and Q properties were inheritable and not due to laboratory manipulation [Isaacs, Gledhill & Andrewes (46), Isaacs (75)]. Strains isolated early in epidemics tended to be Q, and those isolated later to be P, but sometimes mixtures of both were found (66). Passage in mice led to conversion of Q virus to P, perhaps equivalent to the process occurring during spread through a susceptible population. Passage of P virus in the presence of immune serum led to isolation of Q virus (46, 66). Two naturally occurring Q viruses were found to be closely similar to laboratory produced variants [Isaacs (75)]. These findings have been interpreted to mean that P and Q viruses, although similar in antigenic composition, differ in the arrangements of their antigens in the virus particle so that Q strains are able to provoke the production of antibody but combine with it poorly.

Isaacs (76) has suggested that influenza viruses undergo two types of variation, one due to mutation and resulting in the appearance of new antigens not present in the parent strain; and P-Q variation resulting in rearrangement of the antigenic components of the virus.

It has been suggested by many workers that antigenic variants are selected by passage in partially immune populations. The acquisition of a new antigen will allow the virus to spread through the population until immunity is established, when further new antigenic types will arise. Burnet (77) has termed such a process "immunological drift." Its greater capacity for variation would explain the advantage of influenza A over influenza B as an epidemic agent.

There have been many attempts made to mimic in the laboratory this process thought to be occurring in the field. Antigenic variants of influenza viruses have been produced readily by passing the original strain in the presence of immune serum in eggs (39, 46, 78, 79, 80) or in partially immune mice (48, 81, 82). The variants reacted poorly with immune serum prepared against the parent strain, and retained this property on further passage in the absence of serum.

In experiments carried out by passing influenza A strain PR8 serially in partially immune mice, Gerber *et al.* (48, 82) found that a step-wise change in antigenic composition of the virus occurred. A different antigen, present in small amounts in the original strain, became dominant and the original dominant antigen was reduced. The purity of the original strain and of a variant isolated after 21 such mouse passages was tested by the limiting dilution technique. The serological character of the variant was maintained on further passage in normal mice. "Variants of variants" were obtained by continuing the process in mice immunized with the variant strain. The later variants were less able to stimulate antibody production. They gave reasonable protection in active immunity tests against infection by the original virus, although cross-reacting only to a low level with it *in vitro*. The authors warned that *in vivo* tests should always be carried out in assessing the protective potency of vaccine strains. There was a suggestion that later "variants of variants" might become more like the original strain. It was concluded that the antigenic changes were not examples of P-Q variation. Similar experiments were carried out by Magill (81). He considered that the antigenic changes were not necessarily due to mutation, but rather to "rearrangement of existing hereditary elements" under pressure of the imposed immunological conditions.

Beginning with a pure line of the CAM strain of influenza A virus, Edney (80) passed it serially in eggs in the presence of increasing amounts of homologous immune rabbit serum. The virus fluid used for each passage was stored on dry ice. After seven such passages a serum-resistant variant, named SP_7, was isolated and "purified" by the limiting dilution technique. CAM and SP_7 cross-reacted only to a low level in serological tests. The intermediate passage fluids were passed at limiting dilution, and the dominating virus strain in each identified. It was found that apart from the original strain three virus types became successively dominant in the virus population. Each successive strain contained less "CAM-antigen" and more "SP_7-antigen." Each bred true on further passage in the absence of antiserum. It was concluded that the successive variants represented spontaneous mutants, each with a survival advantage over the previous one in the presence of excess CAM antiserum. Similar experiments were carried out three times with the strain CAM in the same laboratory. On two occasions serum-resistant variants were isolated which differed significantly from the parent strain and from each other (79, 80). On the third occasion only the original CAM was isolated (80). Attempts to produce "variants of variants" in this

experimental set-up resulted in a P-Q type of change, or no change at all. A more persistent selective process was probably operating in immune animals. It may be noted that Isaacs *et al.* (46) isolated variants in one passage in eggs, if repeated inoculations of antiserum were given during mutliplication of the original strain.

An interesting finding in Edney's work was the acquisition of pathogenicity for the mouse lung by the variant SP_7, a property not shared by the parent CAM (80). It has been shown that strains of influenza virus unadapted to the mouse lung are more readily inhibited by substances present in normal mouse serum than are adapted strains [Chu (83)]. Variants of unadapted strains have been selected by passing the original strain in eggs in the presence of excess of this "Chu" inhibitor. These variants were resistant to inhibition by normal serum inhibitor and, unlike the parent strain, were pathogenic for mice on primary inoculation [Chu (83); Medill-Brown & Briody (49)]. Edney thought it unlikely that the "Chu" inhibitor in the immune rabbit serum was selecting mouse-pathogenic variants, as normal rabbit serum had little or no neutralizing effect on either parent or variant. It seemed more likely that selection pressure was exerted by the CAM antiserum and the property of mouse pathogenicity was linked genetically to the predominating SP_7. Presumably the possession of mouse pathogenicity offered no survival advantage to virus strains multiplying in chick embryos. Such chance mutations to virulence accidentally selected in immune environments could be of great epidemological importance.

Laboratory experiments have thus supplied interesting parallels to a process of "immunological drift" presumably occurring in nature.

Variations in Pathogenicity and Virulence

In their reactions with host cells, animal viruses may range between being nonpathogenic, when no multiplication of virus occurs and no lesions develop, and fully pathogenic, when multiplication occurs to a high level and extensive damage or death results. "Adaptation" refers to the transition during serial passage of a virus growing in a new host from nonpathogenicity to pathogenicity. Pathogenic viruses may vary in virulence, i.e., in the extent of the lesions they produce. Pathogenicity may be described as a property of one species of virus for a particular animal species or cell population, and virulence as a quantitative difference in pathogenicity between different clones, lines, or strains of a virus for a single host species. For comparisons of virulence, the host, route of inoculation, amount of virus inoculated, and the criteria adopted for measurement of lesions need to be stated. "Attenuation" of a virus refers to reduction in its virulence for a chosen host. Many intermediate grades of reaction between viruses and host cells have been described. Viruses may produce lesions without multiplying, as in the case of Newcastle Disease virus inoculated intranasally into mice [Ginsberg (84)]. This is usually referred to as a toxic reaction. On the other hand, viruses may multiply extensively and produce no lesions or exhibit various grades of

virulence [Hirst (68), Schlesinger (85)]. Inoculation of heavy doses of influenza may result in limited cycles of multiplication and the production of functionally deficient forms of virus, sometimes accompanied by toxic symptoms (86, 87, 88, 89). In certain situations single cycles only of multiplication take place (90, 91). Viruses may also be latent within host cells, i.e., they may be carried without symptoms of infection developing except under unusual circumstances. It is not known whether any of these reactions represent stages in a cycle of development of virus, or stages in the process of adaptation of a virus to a new host. The problems of distinguishing viruses at intermediate stages of development from avirulent variants have been discussed by Schlesinger (92).

The properties of a virus which are of primary practical importance from the point of view of human and animal diseases are its pathogenicity and degree of virulence. In efforts to understand the underlying nature of these properties, attempts have been made in the study of virus variants to correlate virulence with other recognizable properties of the virus strains. Examples of adaptation and attenuation carried out in the laboratory, and variations in virulence observed in the field will be discussed.

Adaptation of influenza virus to the mouse lung.—There has been a great deal of work done on the adaptation of newly-isolated virus strains to laboratory animals. The most intensively studied system so far has been that of the adaptation of influenza viruses to multiplication and the production of extensive lung damage in mice after intranasal inoculation. Recent application of genetic principles has thrown some light on the processes by which adaptation takes place.

On primary inoculation into mice of virus-infected human material lung lesions may be produced. This lung damage is not transferable and may be accompanied by minimal virus multiplication. No lesions are produced until a certain number of passages have been made when the virus multiplies readily, macroscopic lesions develop and death of the animal often results. This pathogenicity for mice is then maintained, whether or not continued passage takes place in mice or in another host such as chick embryos. The primary lesions are referred to as toxic effects.

Toxic effects were first observed by Dudgeon *et al.* (93) with influenza B viruses, and by Sugg (94) with influenza A. Toxic effects were not observed with all strains studied, probably because of differences in the amounts inoculated and differences in toxicity between strains [Henle & Henle (95)]. The first attempts to produce lesions in mice from influenza-infected human throat washings failed, and lesions were produced in mouse lung only if the virus was passed first in ferrets [Andrewes *et al.* (96)]. Francis & Magill (97) were able to demonstrate lesions by the fourth blind passage in mice direct from human material. Despite the fact that few lesions were demonstrated during early passages, it was found that unadapted strains were multiplying extensively in mouse lung [Hirst (68); Davenport & Francis (98)]. Many workers have found differences in other properties besides

virulence in unadapted and adapted strains of common origin. Some adapted strains have been shown to have a shorter lag period before detectable multiplication begins (98), to multiply more rapidly (99, 100), and to a higher titre (98) than unadapted strains. They may differ in the heat sensitivity of their haemagglutinins (101), in their power to provoke antibodies in mice (102), and in their reactions with inhibitors (83, 101, 103, 104). Changes in antigenic constitution have been observed after adaptation (68), but do not necessarily occur (99).

Assessment of the properties of the virus and the host at different stages of adaptation showed that the appearance of lesions was correlated with increase in the production of infective hemagglutinating virus, disappearance of inhibitor in the mouse lung, and acquisition by the virus of the property of resistance to inhibition by β or "Chu" inhibitor (83, 103, 104, 105). It was concluded that variants or mutants had arisen which were better able to multiply in the mouse lung than the original strain, possibly because they were resistant to inhibitors present in the mouse lung (83).

Burnet & Lind (79) studied the process of adaptation of the A strain CAM. The virus was passed 20 times serially in mouse lung, bronchial washings being used to initiate each passage. Lung lesions appeared at the eighth passage and increased from then on. Increase in haemagglutinin was detected at the twelfth passage, and all mice inoculated with the standard dose died from the seventeenth to the twentieth passage. Limiting dilution titrations in eggs were carried out on material used for the eighth and ninth passages. Lines of virus were isolated with two distinct grades of virulence. There was a progressive change, with increased mouse passage, to increased virulence, and all lines isolated from the twentieth passage were of high virulence. The degree of virulence was inherited but tended to decrease on repeated passage in eggs. Similar studies were carried out by Ledinko (106). After adaptation the virus was of high stable virulence and differed from the unadapted strain in behavior towards inhibitors and in multiplication rate. Limiting dilution titrations on intermediate passage material yielded virus strains intermediate in properties between the unadapted parent and the adapted derivative. In the intermediate strains higher growth rate was correlated with degree of lung damage caused, rather than with increased resistance to inhibition. Some fully virulent strain were isolated from early passage fluids. Ledinko concluded that mutation and selection were primary factors in adaptation, but that environmental factors could exert an influence in the Lamarckian sense.

Ledinko & Perry (105), in attempts to use as starting material a virus closely related to the human pathogen, first passaged a freshly-isolated virus at limiting dilution in the amniotic cavity of the chick embryo. They found that lesions were produced on primary inoculation into mice and were accompanied by the production of hæmagglutinating noninfectious virus. The authors were using a relatively small dose of virus and thought the lesions were not toxic in origin but rather due to some property of the virus

related to pathogenicity for humans which was gradually lost on passage. Prolonged passage in the chick embryo before inoculation into mice resulted in reduced capacity to multiply and produce lesions. Repeated passage at limiting dilutions did not maintain the virus indefinitely in its original form, but a more rapid change occurred if the virus were passed at lower dilutions.

It is interesting that mouse pathogenic variants have been isolated from unadapted strains without passage in mice, first by selection in eggs for resistance to inhibitor and secondly by selection in eggs for antigenic differences. Chu (83) isolated inhibitor resistant variants by inoculating sensitive strains into eggs in the presence of normal mouse serum which contains β or "Chu" inhibitor. One such variant produced lesions in mice on primary inoculation. The pneumonia produced was probably toxic in origin, as it was not transferable from mouse to mouse. Chu concluded that, although increased resistance to inhibitor accompanied adaptation and possibly favoured multiplication in the mouse lung, it was not a primary factor in determining pathogenicity. Similarly, by inoculating into eggs an inhibitor sensitive ("S") strain of influenza virus in the presence of excess ox serum, a potent source of inhibitor, Medill-Brown & Briody (49) were able to isolate inhibitor resistant "R" clones which were pathogenic for mice on primary inoculation. They considered that R virus particles represented spontaneous mutants appearing with a calculated frequency of $1/10^8$. Although not fully pathogenic for mice, R lines adapted more rapidly than S lines. During the process of adaptation, the virus populations were examined and found gradually to be dominated by R lines. The authors concluded that, during adaptation to mouse lung, two types of mutation took place. The original S population gave R mutants which grew more rapidly in mice because they were unaffected by inhibitors, and mutated more readily to highly virulent forms. Edney's mutant SP_7 (80) derived from strain CAM after passage in eggs in the presence of anti-CAM serum, has already been described. SP_7 was resistant to neutralization by anti-CAM serum and pathogenic for mice on primary inoculation. An intermediate strain serologically very similar to SP_7, was equally virulent. The quality of pathogenicity seemed to be linked to the SP_7 antigenic character.

In searching for an explanation of the process of adaptation, Davenport (107) assumed that variants were present in the original population and possessed either a survival advantage or a greater potential for mutation to virulence than the original virus. Workers who began their experiments with pure lines of virus have concluded that adaptation consists of spontaneous mutation with selective survival of more virulent variants. Dulbecco (43) has suggested successive mutations with an additive effect. Selective pressure applied by the environment could account for the sudden appearance of virulence (45, 49, 80), but did not seem sufficient explanation for the gradual increase in virulence in a favourable environment, and decrease in a neutral environment (79, 105, 106). On the basis of adaptation experiments and recombination experiments involving the transfer of virulence from one virus

strain to another, Burnet (108) has postulated mutation, followed by smooth replication of "virulence genes," which multiply independently of the rest of the virus genetic apparatus. In a favourable environment, the number of virulence genes would increase progressively, and in an unfavourable environment would decrease. In other words, virulence may be increased under the influence of the environment, in analogy with the production of adaptive enzymes.

Attenuation of poliomyelitis viruses.—The primary aim in the search for variants of poliomyelitis viruses has been to obtain attenuated strains which have lost their virulence for humans while retaining their antigenic potency. A certain amount of success has already been achieved.

The three immunological types of human poliomyelitis virus, I, II and III, have been adapted to growth in mice and cotton rats inoculated by the intracerebral or intraspinal routes. In many cases adaptation to pathogenicity for rodents has been shown to be associated with reduction in virulence for monkeys or chimpanzees (109, 110, 111, 112). Adaptation of the viruses to chick embryos (113) and tissue culture of extraneural tissues (27, 41, 114, 115, 116, 117, 118) has also led to reduction in their virulence for primates. Variants of Types II and III, avirulent for monkeys, have been isolated in tissue culture from rectal swabs of healthy children [Ramos-Alvarez & Sabin (119)]. Changes in virulence for mice of a mouse-adapted Type III strain were brought about by further passage in mice resulting in increased virulence, or further passage in tissue culture resulting in decreased virulence [Li & Schaeffer (116)]. However, in experiments with a mouse-adapted Type I strain, no changes in virulence were observed after 43 consecutive tissue culture passages [Li & Schaeffer (120)]. Experiments have been carried out in feeding attenuated strains to humans. The strains were avirulent for monkeys and chimpanzees inoculated by neural or extra-neural routes. Attenuation was achieved by adaptation to rodents (121, 122), chick embryos (123), or by isolation in monkey kidney tissue culture (124). The human volunteers developed antibody but no illness, and in some cases excreted virus in the faeces. The properties of the virus remained stable after human passage [Roca-Garcia *et al.* (123)]. However, recent experiments of Dane, Dick, and co-workers (125, 126, 127) have shown that excreted virus may, in fact, differ from the vaccine virus fed to the volunteers, and may cause paralysis in monkeys.

Only recently have attempts been made to ensure that pure lines of virus have been used, and to apply genetic principles to the problems of attenuation in poliomyelitis viruses. Li & Schaeffer (120) and Sabin and co-workers (41, 128) have used the limiting dilution technique, and Dulbecco & Vogt (27, 118) the plaque technique. Variants were readily selected in monkey kidney tissue culture by passing large amounts of virus at short intervals (41). Use of monkey testis tissue culture appeared to favour the appearance of variants [Li, Schaeffer & Nelson (129)]. Sabin (128) emphasized that, in defining virulence, the host and route of inoculation must be stated. Virulence

in monkeys or chimpanzees observed after extraneural administration was independent of virulence shown after intracerebral or intraspinal inoculation. Spinal and cerebral virulence were not necessarily correlated, nor were virulence for mice and monkeys. Some attenuated strains selected in tissue culture appeared to revert to the virulence of the parent strain after inoculation into monkeys. Sabin pointed out that such virulent strains often differed in other properties from the original strain, and represented rare mutants, with no survival advantage in tissue culture, selected in a favourable environment.

Attenuation of strains selected in tissue culture by limiting dilution and plaque techniques has been ascribed to mutation [Sabin (128); Dulbecco (118)]. Since virulence for mice was not necessarily linked with lack of virulence for monkeys, the explanation of attenuation of the virus for monkeys might well be reduction in "virulence genes" for monkeys after passage in mice. A similar explanation might hold for the changes in virulence for mice observed after passage in mice and tissue culture (116). Dulbecco & Vogt (27) have emphasized that the role of the host cell needs defining as host cells might apply mutation pressure as well as selection pressure. Clarification of the situation is expected with increasing application of the precise plaque technique and use of pure lines of host cells in the study of variation in poliomyelitis viruses.

Variations in virulence of myxoma virus.—The recent epizootics of myxomatosis provide an excellent example of the way in which a change in virulence of a pathogenic organism has influenced the pattern of a disease.

When the myxoma virus was first successfully released in Australia in 1950, 99.8 per cent of the rabbit population was exterminated. Infection was carried by mosquitos or spread by contact. In later years milder epizootics occurred, only 90 per cent of the rabbits died, and many chronically ill animals were observed [Myers, Marshall & Fenner (130)]. Attenuated strains of virus were recovered in the field in Australia (131, 132) and also in England (133) and France (134). When examined in quantitative tests for virulence in rabbits, the attenuated strains were found to differ from the original strains released in the field in the types of lesions produced and the mean survival time of infected rabbits (15, 135, 136). Mykytowycz (16) established pure lines of virus by isolation and passage from single pocks on the chorioallantois or primary lesions on the rabbit skin, and showed that reduced virulence was inheritable and not due to a mixture of strains, as suggested by Jacotot *et al.* (137). The severity of the disease was influenced by the mode of infection, bites by an insect vector being more effective than contact infection, and by climatic conditions (16). The dominance of the attenuated strains over the original highly virulent strain was accounted for by the greater survival time and longer infectious state of rabbits infected with less-virulent strains, and consequently by more efficient mosquito transmission [Fenner, Day & Woodroofe (15)]. Day *et al.* (138) could find no evidence for multiplication of virus in the insect vector, although the very long

time of survival of virus in mosquitos (up to 220 days) led Andrewes and co-workers to suggest that perhaps limited multiplication was taking place (136). Nine serial mosquito-bite passages, where the virus was maintained successively in the rabbit for one week and mosquitos for two weeks, had no effect on the pathogenic behaviour of the standard virulent strain (15). Repeated passage in rabbits did not increase the virulence of an attenuated strain [Jacotot, Vallée & Virat (139)].

Thus stable variants of myxoma virus have arisen in the field, probably by a process of mutation, and have replaced more virulent original strains because of their greater fitness for survival and spread.

Miscellaneous Variations

Variants of the Lee strain of influenza virus, differing from the original strain in rate of elution from red cells, have been obtained by single treatments of the original virus with lanthanum acetate or ultraviolet light [Björkman & Horsfall (140)]. The evidence indicated that selective inactivation of the original strain occurred with survival of the variant which was already present in the original population. Serial passage of mumps virus in eggs in the presence of inhibitory amounts of bacterial polysaccharide led to the appearance of a variant strain resistant to inhibition by the polysaccharide. If a large amount of virus was inoculated, the variant was obtained in one passage. The parent strain reappeared after further passage without polysaccharide. A mixture of sensitive and resistant strains was obviously present at the beginning of the experiment [Ginsberg & Horsfall (141)]. Goldman & Hanson (142) isolated heat-resistant mutants of Newcastle Disease virus, which bred true on further passage at limiting dilution, after subjecting the original strain to repeated passage after short heat treatment.

Careful experiments by Dulbecco (118), utilizing the plaque technique for isolation of variants and purification of virus lines, have clarified the origin of some poliomyelitis virus variants. The starting material was a pure line of poliomyelitis virus Type I which produced plaques on monkey kidney monolayers, was heat sensitive in a standard test, and fully pathogenic for monkeys. A mutant (*d*) was isolated which produced plaques appearing two days later than those of the original strain on acid media. The mutant was reduced in virulence for the monkey. The *d* virus gave rise to mutants of the wild type (d^+), selected again by plaque formation on acid media. Fluctuation tests showed that the mutation to d^+ was not induced by the acid selective environment, but produced during growth of the *d* stock. From the wild type was isolated another mutant, *t*, resistant to heat but showing the same plaque and virulence characters as the wild type. This mutant was selected by successive heating and purification through plaque passages. Evidence from recombination experiments suggested that mutation to heat resistance took place in two stages, mutation to intermediate resistance occurring first. Thus different mutations from the wild type have been described which were independent of each other and bred true for at least

100 generations. The frequency of mutations $d \rightarrow d^+$ and of $t^+ \rightarrow t$ was of the order of $1/10^6$. Physiological changes simulating the true genetic changes were observed.

Variations in Animal Viruses as a Result of Genetic Transfer

Mixed infection of cells with two related but dissimilar virus strains has been shown to yield new types of virus combining characters of both parents. Most of the new types bred true on further passage. Although certain virus properties were always linked, the character of virulence tended to behave independently of other properties, with different grades of virulence distributed among the progeny of genetic crosses. Doubly-neutralizable viruses (i.e., single virus particles carrying antigenic characters from both parent strains) and heterozygotes yielding different strains on further passage have been demonstrated. Recombination of virus characters has been observed in influenza, herpes, and poliomyelitis viruses. The subject has been recently reviewed by Burnet (44).

Fibroma virus mixed with heated noninfective myxoma virus and inoculated into rabbits produced myxoma-like lesions. Serial passage from these lesions gave active myxoma virus [Berry & Dedrick (143)]. The virulence of the new myxoma virus was never greater than that of the myxoma virus which supplied the transforming substance Smith (144). The nature of the transforming substance has not yet been determined.

Conclusions

Some of the phenomena encountered in the study of variations in animal viruses have been explained, and some of the processes by which variants have arisen elucidated. Some physiological variations, resembling true genetic changes but dependent on immediate environmental conditions, have been observed. True-breeding variants, present in the original population and of obscure origin, have been selected in favourable environments. When the starting material was a pure line of virus, mutants arising spontaneously were selected because of a survival advantage or because of the distinctive appearance of the pocks or plaques they produced. Drastic variations have been shown to result from successive mutations, additive in effect. Selection of mutants of low frequency was avoided by passing virus at limiting dilution when only the major component of the virus population was reinoculated. Passage of large amounts of virus encouraged
30 the selection of variants. In certain cases, variations could not be attributed entirely to mutation and selection. Increased or decreased virulence resulted from passage of virus even at limiting dilution.

The unpredictability of results in experiments on virus variation is compatible with a theory of such variations arising by a process of chance mutation. Reversion of a mutant to the parent type could occur if virus lines were not purified, or if further mutations occurred. The reasons for linkage of changes in properties apparently irrelevant for survival of the virus with variations favouring survival have not yet been adequately explored.

Future experiments with animal viruses will no doubt be carried out on pure lines of virus, certainly arising from single virus particles, infecting pure lines of host cells [Puck & Marcus (145)]. Examination of the progeny of a single particle infecting a single cell is now possible [Dulbecco & Vogt (146)]. Recombination experiments will lead to further information on linkage groups and on the number of mutational steps required to bring about a change from one virus type to another. Already some information is available relating chemical composition of animal virus particles to their functional behaviour [Ada (147)]. It may not be too much to hope that further examination of the nucleic acid and protein components will lead to greater understanding and perhaps some degree of control of variations in animal viruses.

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REVIEW OF THE MICROBIOLOGICAL AND IMMUNOLOGICAL LITERATURE PUBLISHED IN 1956 IN THE U.S.S.R.[1]

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Introduction

As in last year's review, the author's aims are: to inform but not to discuss, and to be as complete as possible. Limited space imposes the obligation of mentioning only a part of the published material and therefore some subjects will again be omitted, such as: fungi, yeasts, parasitology, clinical aspects of infectious diseases, industrial applications of microbiology, use of antibiotics in practical medicine, and most of the practical methods of diagnosis. In many instances, the author was obliged to cite only a few publications on a given question or in a certain domain, and the absence of citations of works on some problems does not always imply lack of publications on the subject.

The main periodicals which publish articles on the subject of this review have been cited in last year's review. New journals, one specialized in virology, *Voprosy Virusologii*, and another devoted to antibiotics, *Antibiotiki*, have appeared but the author has had no opportunity to consult them. Some of the periodicals in the Russian language began, in 1956, to add summaries, mostly in English but some in French or in German. Among those summaries which concern this subject, some are to be found in *Microbiologia*, *Biokhimia* and *Problemy Tuberkuloza*. The *Doklady Akademii Nauk S.S.S.R.* publishes only translations of the titles of the articles.

The general trends seem to be the same as in 1955. Most of the published articles are concerned with practical aspects of microbiology, i.e., the applications for an immediate practical purpose. But, again, several more general questions have been the subject of "contributions to the discussion" of a problem. One of such questions which occupies an important place is the role of the nervous system in infectious pathology and in immunology. Therefore, this question has been treated in a separate section. The other problems are grouped in the same order as in the previous review.

The *Journal of Microbiology, Epidemiology* and *Immunobiology, (U.S.S.R.)* has largely increased the number of articles per issue but some of those articles are still only summaries of accomplished work, generally without description of methods and without discussion of results. The *Micro-*

[1] The survey of the literature pertaining to this review was concluded in January, 1957.

[2] The author expresses his thanks to Mrs. Nina Grabar and Miss Ludmila Grabar for their contribution in assembling the bibliography, and to Mrs. Helene Miller for her help in correcting the English text of this review.

biologia publishes mainly works on soil microbiology and on agricultural applications of microbiobiology.

The delay in publications seems not to have changed and many, if not most, of the articles under review were submitted in 1955.

Systematics, Morphology, Variations

A new Gram-positive bacterium which infects Siberian silkworms has been isolated and its morphological and cultural properties have been described by Talaev (1) who proposes to name it *Bacillus dendrolimus*, n. sp.

Buckwheat plants may present a kind of mosaic similar to that observed on the sorrel; it appears on young plants and particularly in a moist atmosphere (2). A specific phage acting on *Clostridium acetobutylicum* and stopping the acetone-butylic fermentation has been described and some of its properties studied; the appearance of some phage-resistant cells has been observed (3). Streshinskii (4) has studied the "individuality" of the bacterial cell and admits that it has periods of development and senescence, with a "normal death," but stresses that the period of early development which would correspond to the embryonic state, still awaits an experimental approach.

Cytochemical examinations of typhoid and paratyphoid bacteria showed that polar inclusions, characteristic for O and for Vi-antigens, contain polysaccharides which seem to be in complexes with nucleic acids (5). The modifications of the cells of *Salmonella suipestifer* under the action of sonic waves has been studied by electron microscopy (6).

Draganov (7) supposes that the "Filamentous corpuscles with enlargements" which one observes in cultures of influenza viruses correspond to the vegetative form, and the elementary bodies to the primitive form of this virus.

Continuing his research on the polyhedric viruses of insects, Gershenzon (8) has observed that a penetration of the virus in the nucleus protects it for several hours against a new infection, but after this period a new invasion is again possible. When the polyhedres are introduced in the hemolymph of the insect, the virus is liberated from the polyhedres and penetrates into the cells. The hemolymph does not contain free viruses at that moment and the infection of a new animal can only be accomplished by using entire cells.

In the cultivation of aucuba-mosaic virus on *Nicotiana glutinosa* under different conditions (temperature, time, darkness, light) Sukhov & Kapitsa (9) observed a rapid appearance of highly infective material; they think that it is due to a transformation of an accumulated noninfective substance (which would be the vegetative phase) into virulent virus particles.

The mosaic of the red clover is due to a complex of two viruses: Pea-mottle virus and Pea-wilt virus, which, under natural conditions, are always found on the clover. A second virus-induced mosiac of clover is due to the Vein-mosaic virus of red clover, Osborn, which produces other effects (10).

Electron microscopy permits observation of the appearance of filamentous structures in preparations of dysentery phages during their prolonged

storage. These long threads are associations of round particles and this structural modification seems to parallel the loss of phage activity (11).

As in last year's review and for the same reason, the publications on variation, dissociation, adaptation, and mutation will be considered together. About 20 articles on these topics were published in 1956. Some of them were concerned with the theoretical question of the formation of new species and its implication in medical microbiology, others with the formation of variants useful for practical applications (fermentations, production of antibiotics, etc.). Only a few publications containing experimental results will be cited. In a series of articles on *Salmonella typhosa*, isolated from patients at different stages of typhoid fever, the authors claim that they observed variations in the contents of O and Vi-antigens and in the virulence of coprocultures and of cultures isolated from the urine, but it seems that no regularity in these variations could be established (12a).

Quantitative paper chromatography and ultraviolet photometry were used for a comparative study of RNA and DNA composition of original strains and variants of microorganisms of the intestinal group; strains obtained by cultivating *Escherichia coli* on killed *Salmonella paratyphi B* showed a modification of the DNA composition corresponding to new hereditary characters, whereas the RNA composition remained constant (12b).

An atoxinogenic variant of *Corynebacterium diphtheriae*, PW8, has been obtained by prolonged cultivation in a medium containing citrate (13). Stovbun (14) has observed the transformation of *E. coli* into coccal forms by cultivating them in acid medium (pH:4, 1–4, 2); only few of which could return to the original morphologic aspects. The cultivation of *Bacillus mesentericus* on a "bread medium" has resulted in the formation of a new type which behaved as a stable variant (15). The introduction of killed typhoid or dysentery bacilli into an isolated part of the intestine of rabbits has produced some variations in the biochemical properties of the saprophytes. When living bacilli were introduced some serological modifications of both the saprophytes and the introduced pathogens were noted, but all these modifications have been assumed to be only superficial (16). Some modifications in the nucleic acid content (mainly DNA) of *E. coli* under the action of an antibiotic agent (grisemin) have been observed; the rate of multiplication of the resistant variant was different from that of the original strain, and the antibiotic had a stimulating activity on the resistant strain (17a).

Biochemically-deficient variants of *E. coli* and *S. paratyphi B* requiring methionine or vitamin B_{12} were studied using radioactive S^{35}. The results show that in order to increase independent methionine synthesis these strains require very large amounts of B_{12}, a thousand times larger than those which are necessary when they grow on a minimum medium at the expense of independent methionine synthesis (17b).

When *Brucella* are submitted to the action of a type-specific phage, two kinds of secondary cultures have been observed. One, which appears in 30 to 40 hr., gives strains identical with the initial strain. The other, which ap-

pears only 4 to 6 days after apparently complete lysis of the culture, produces a new variant which has many properties different from the original strain, including resistance to the original phage, but which can be lysed by a polyvalent phage (18).

The influence of photo-reactivation on the mutations induced by ultraviolet rays in *Actinomyces globisporus streptomycini* has been investigated, using various dosages of ultraviolet light; morphological modifications and production of streptomycin have been chosen as tests. A large increase in the dose of irradiation followed by photoreactivation by visible light has provoked a higher frequency of mutations with altered synthesis of streptomycin, whereas the frequency of morphological mutations is modified by smaller doses which have no effect on the frequency of the appearance of mutants producing no streptomycin (19).

Sukhov & Kapitsa (20) found that the potato X-virus shows variations in mixed infections with the tobacco mosaic virus; they suppose that these variations can be due to the mutual metabolic influences of these two viruses when they multiply in the same cells.

Studies on the so-called "filterable forms" of microbes have been less numerous this year. The use of symbiotic microbes as nutrients for the regeneration of such forms has been studied. Of 145 tested strains of *Sarcina* and staphylococci isolated from the air, 34 per cent gave positive results in the regeneration of filterable forms of *E. coli*, but some cultures of filterable forms of *Sarcina* have shown many analogies with the early regenerative forms obtained with *E. coli* (21). The respiration and the fermentation activity of "regenerated" strains of *S. Paratyphi B* (*B. breslau*) (22) and the reversion of secondary cultures of *Salmonella gallinarum* obtained from filterable forms, to the initial strain in one-day-old chickens have been studied. In this last work it is stated that among 81 secondary cultures, 73 made a complete reversion to the initial properties and that due attention has been paid to the possibility of a pre-existing infection in the chickens (23).

Biology of Microorganisms

Constituents, metabolism, enzymatic activity.—Kuznetsov has envisaged the possibility of a synthesis of organic substances under the influence of natural radioactivity, and his calculations have shown that the quantity of energy necessary for the synthesis of one μg. of organic substance in one liter from CO_2 and H_2O is of 0.0058 μc., assuming that the radioactivity is transformed into heat. On the other hand, the natural waters have an activity of only 50×10^{-9} μc. From this quantity, some hundred thousand times smaller than the preceding one, he concludes that such radiosynthesis does not exist under natural conditions (24a).

The chemical composition of *Brucella abortus*, *Brucella suis* and *Brucella melitensis* has been studied. The contents in total lipides, in reducing substances, and in nucleic acid P have been determined and the presence of 12 different amino acids has been established (24b). Studies on the constituents

of diphtheria bacilli have shown that they contain numerous fatty acids and neutral lipides. Many fatty acids have been isolated; among them, some are branched and may contain 18 to 20 carbons; others are unsaturated high-molecular components (31 to 32 carbon atoms) (25). The polysaccharides of the same microorganism have been studied using paper chromatography and different methods of hydrolysis (26a).

The synthesis of polysaccharides from glucose-1-phosphate by *Clostridium perfringens* and *Clostridium oedematiens* is enhanced by starch and maltose, but not by sucrose and lactose. *C. oedematiens* synthesizes an intracellular starch-like polysaccharide which consists of glucose molecules and is split by salivary amylase and by potato and bacterial phosphorylases. A similar polysaccharide is formed by *C. perfringens* only on dextrin-containing media (26b).

Suspensions of *C. oedematiens* catalyse phosphorylation of glycerol at the expense of ATP. The pH optimum of the glycerokinase is 6.9 to 7.0 and it is activated by Ni, Co, and Mg ions, and strongly inhibited by Zn and Ca ions. This enzyme was found as well in type A, which metabolizes glycerol, as in type B which is not adapted to glycerol (26c).

Meisel and his collaborators (27) have observed an increase in the yield of ergosterol from yeasts submitted to the actions of ultrasonic waves, but not under the influence of other physical agents such as ultraviolet light, temperature, and x-rays.

The synthesis of carotenoids in *Sarcina* has been studied by Bezborodov (28) in various conditions (pH, presence or absence of air); this synthesis takes place when the growth of the cell is completed; diphenylamine acts as an inhibitor, and anaerobiosis diminishes it. The light absorption maxima of the pigment are situated at 415,440, and 470 mμ, and its melting point is 148 to 150°C. Variants of *Shigella paradysenteriae* (Flexner) containing a yellow pigment can be obtained when these microbes are cultivated in certain conditions, and particularly when the culture is made on leucocytes; this pigment is a carotenoid (29). Among 200 strains of *Pseudomonas pyocyaneus*, 5 produced abundantly a red pigment possessing antagonistic properties; it is soluble in water but not in chloroform, dichlorethane, xylol, ether, etc., and does not become yellow in acid media, as does the pyoxanthose (30).

Purple bacteria (a strain analogous to *Rhodopseudomonas palustris*) can grow in the presence of light on media in which carbonate has been replaced by organic substances, particularly by organic acids (31).

Studies on *Streptococcus lactis* have shown that this organism utilizes phosphoric acid when it grows on milk, that it posessses a lactase and that glucose-1-phosphate and glucose-6-phosphate are normal intermediate products in the degradation of lactose by this microbe (32).

Paper chromatography has been used in studies on the amino acid content of media on which various strains of *E. coli* have been cultivated, and differences have been noted (33).

A review on bacteria which decompose protopectin has been published by Lambina. The protopectinase has been found not only in phytopathogenic bacteria but also in certain saprophytic soil microbes (*Bacillus polymyxa, Alcaligenes radiobacter*). Their possible interference in agricultural practice has been stressed (34).

DNA stimulates the growth of yeast more than RNA; streptomycin inhibits this effect (35). Using paper chromatography and trichloracetic acid precipitation, Smirnova (36) has observed that the addition of nucleic derivatives to autolysates of washed cells of *C. diphtheriae* modifies the activity of their proteolytic enzymes; the addition of nucleoproteins isolated from the same microorganism seems to increase the synthesis of proteins, whereas the addition of yeast nucleic acid provokes an activation of proteolysis.

Studies on deaminases of purines and nucleic acids of several strains of *E. coli* and *B. breslau* have been made by Rashba & Tsinkalovskaia (37), and the modifications of the nucleases in the same microorganisms and their variants, by Galkina (38). The variants were obtained by cultivating *E. coli* on media containing "complete antigen" of *B. breslau*. Generally, the ribonuclease was more active than the deoxyribonuclease; no significant differences among the studied strains were observed in the case of ribonuclease, whereas the activity of the deoxyribonuclease of the variants was more or less similar to the activity of this enzyme in the *B. breslau* strain.

Several papers have been published on the stimulating effect of simultaneous cultures of two different microorganisms, such as the action of *Streptococcus lactis* (or of the filtrate of its culture) on the growth of *Streptococcus paracitrovorus*, the action of aerobic bacteria on *C. pasteurianum*, the use of synergic cultures in industrial installations, etc. Thus, for example, Suslova (39) used filtrates of cultures of *Bacillus mesentericus fuscus* and *Bacillus vulgatus* in cultivating *C. diphtheriae* and reports that toxinogenesis was promoted by these filtrates because she observed more intense formation of precipitation bands in Ouchterlony's test of double diffusion in gels.

The action of bactericidal and antibiotic substances.—Among publications on disinfection only three will be mentioned, most of the others being concerned mainly with classical aspects of this problem and with practical applications (use of different disinfectants, aerosols, etc.). An apparatus for the disinfection of different materials such as books, packs, etc., by high frequency current has been elaborated upon. Some useful observations have been made with it and seem to justify its practical use, namely for bales of rag (40). Studies on the residual microflora in tinned meat and fish showed that some sporogeneous microbes (mainly *Clostridium putrificum* and *Clostridium sporogenes*) may resist heating at 125°C. during 35 min. (41). Different qualities and preparations of tea have been examined and have shown an antiseptic activity against *Shigella paradysenteriae;* this activity was increased during prolonged conservation of tea extracts and was not impaired by filtration or sterilization (42).

In simultaneous cultures of *Mycobacterium tuberculosis* with certain bac-

teria, fungi or yeasts in an N-poor medium, Shiller has observed the formation by these microorganisms of substances (lysins) capable of lysing mycobacteria. This transformation into antagonists is maintained even when these microorganisms are cultivated in pure culture. Tuberculous guinea pigs treated with crude preparations of these lysins showed a certain degree of resistance. These substances are not toxic and Schiller hopes that when purer preparations are obtained they may be tried as a therapeutic agent (43).

A substance (named "diphtherin" or D-substance) possessing an antibacterial activity against *Hemophilus pertussis* and *M. tuberculosis* has been found in acetone extracts of old broth cultures of *C. diphtheriae*, PW8 (44). Another antibacterial substance has been found in the fulvionic acid fraction of soil humus and a certain degree of purification has been achieved (45). *Trichoderma pers* contains volatile antibacterial substances active on Gram-positive and Gram-negative microbes and on some fungi (46).

Comparative cytological and physiological studies on the producers of antibiotics: *Act. globisporus*, *Streptomyces aurefaciens* and *Act. rimosus* have shown that during the first phase, characterized by an intense growth of the mycelium, the protoplasm is basophillic and rich in RNA, but there is practically no production of antibiotic substances. Whereas, in the second phase, the growth and the formation of RNA is diminished but the quantity of DNA is increased and large amounts of the antibiotic substance are synthesized (47). The antibiotic activity of *Pseudomonas fluorescens liquefaciens* in the R form is said to be higher than in the S form (48).

Several papers have been published on the mechanism of the activity of different antibiotic substances. For example, Korotiaev (49) found that chloramphenicol inhibits proteolysis and the carbohydrate metabolism of bacteria (this last at a stage preceding the formation of acids); it has no action on lipases and does not block sulfhydryl groupings. Such substances as riboflavin, thyamine, pyrodoxin, nicotinic acid and nicotinamide, acetylcholine, cystine, and aspartic acid provoke no inhibition of its antibiotic activity.

Many other publications were concerned with the treatment of different diseases by various antibiotics or combinations thereof, with the use of medicamentous sleep during such treatments and with the formation of antibodies in antibiotic-treated patients.

The appearance of strains resistant to one or more antibiotics has been the subject of several publications and the higher frequency of such strains in places where these substances are used has been stressed. Morphological and biochemical properties of resistant strains of different bacteria have been studied. Sazykin (50) insists on the observation that, in many cases, the albomycin-resistant strains of certain bacteria lose some of the properties which are generally used for the identification of these microbes. In a series of publications Moroz (51) described the results of his investigations on the action of the antibiotic grisemin on staphylococci and intestinal bacteria.

The resistant strains showed differences from the original strain in their morphology, their rate of growth, and several biochemical characteristics. The resistance to this antibiotic substance was maintained by the strain even after 80 passages on ordinary media without any addition of the antibiotic. The resistance to grisemin was accompanied by some resistance to other antibiotics.

Differences in the structure of tubercle bacilli submitted to the action of streptomycin and of phtivasides have been observed using electron microscopy and have been attributed to differences in the mechanisms of their action on the cells. In some cases loss of acid resistance has been observed (52).

The healing of lesions in experimental tuberculosis during treatment with streptomycin, PAS, or phtivasides has been followed by histochemical examinations of the distribution or accumulation of nucleic acids in the tissues. PAS, and particularly the phtivasides, increase this accumulation, whereas in streptomycin-treated cases they appear only slowly (53).

Microbiology of soil and water.—As in 1955, a great number of publications has been devoted in 1956 to this question, most of them published in *Microbiologia*. Some are general reviews or "contributions to the discussion," as, for example: "Summaries of the Discussion on the Objectives of Soil Microbiology" (A. A. Imshenetskii); "The Physiology and Biochemistry of Nitrifying Microorganisms" (E. L. Ruban); "On the Theory of Chemoautotrophy" (I. N. Sorkin); "Does Chemosynthesis Exist in Iron-bacteria and in Nitrifying Bacteria?" (V. O. Kalinenko); and criticism of this last article by Shaposhnikov and by Zavarzin ("On the Possibility of Heterotrophic Metabolism in Nitrifying Bacteria"); all of them appeared in this journal.

The microflora of rocks in high mountains has been studied, and Krasilnikov, in a general review, insists on its role in nitrogen fixation. The oligonitrophilic bacteria which grow on lichens and among algae on the rocks, fix during the summer (6 months) 37 to 80 kg. of nitrogen per hectar of soil covered with lichens. The nitrogen components formed in the rocks are washed out by rains and transported into the valleys (54).

Fedorov & Kalinskaia (55) found that nitrogen fixation by *Azotomonas fluorescens* is proportional to the chemical energy of the substrate used and corresponds to 2 to 3 mg. of N_2 per Kcal. They assume that the fixation of N_2 is a function of the growth of the organism and that the same general laws govern N-fixation by this microbe as by *Azotobacter agile*. Certain soil *Actinomycetes* are capable of fixing a small amount of N_2 from the air, but this fixation is a very slow process (56).

Series of publications concern the use of bacterial fertilizers and particularly of *Azotobacter* and phospho-bacteria in agriculture. In a general review on this problem Rubentchic (57) insists on their utility when convenient conditions for their growth exist or have been created. Experimental field results obtained by Krongauz (58) show that in most cases the use of

Azotobacter had no effect, mostly because this organism can not grow in insufficiently moist or in acid soils. The concentrations of various salts which inhibit the growth of this microbe have been studied by Sushkina. She assumes that antagonistic actions of different cations may result in creating favorable conditions for its development (59).

In the presence of small amounts of carbon compounds the multiplication of *Nitrosomonas* and the oxidation of ammonium salts are slowed down. When large amounts of sugars are added, nitrification is completely stopped, but this activity is restored when the cells are transferred to an inorganic medium. Ruban assumes that in natural conditions this organism persists in a latent state as long as its environment is rich in easily assimilable organic substances (60). Imshenetskii and his collaborators (61) have continued their investigations on nitrification in cell-free autolysates of *Nitrosomonas.* They found that the oxidative system is relatively resistant to heat, short boiling of the autolysates does not inactivate it. Hydroxylamine is oxidized to nitrite by these extracts and it is assumed that this substance is an intermediate product in the transformation of ammonia into nitrites (62). Attempts to obtain analogous cell-free filtrates from *Azotobacter* have met technical difficulties, and such filtrates, as well as destroyed cells, were devoid of the capacity to fix atmospheric nitrogen (63).

The role of bacteria in the transformation of phosphates in the soil has been investigated. Uvarova (64) has studied the decomposition of tricalcium phosphates by bacteria. Tulaikova (65) has observed the growth of various soil microbes in contact with superphosphates and has demonstrated differences in their behavior. Smallii (66), using phosphate labelled with P^{32}, studied its absorption by various microbes of the rhizosphere and its penetration into the roots of wheat.

Forty-one different species and 22 sub-species of microorganisms have been identified in the microflora of wheat roots, and some of their properties useful for the plant (nitrogen fixation; phosphorus metabolism; formation of substances stimulating growth of the roots) have been established (67). The rhizosphere of trees has been investigated, the number and the distribution of various microorganisms, including *Actinomycetes,* their seasonal modifications, etc., has been envisaged (68). The formation of the mucorhizis of the oak is claimed to be due to *Hebeloma crustiliforme* (69).

Experimental introduction of four phytopathogenic bacteria (*Erwinia aroidea; Erwinia carotovora; Phytomonas citriputeale* and *X. vesicatoria*) into nonsterilized soil has resulted in a rapid loss of their capacity to produce infections. Most of the reisolated strains were not agglutinable by specific sera and were nonpathogenic; some of those which were still virulent showed modified agglutination or physiological properties. It is assumed that the pathogenic microbes are partially destroyed by antagonists and partially transformed into variants which may still possess some pathogenicity (70).

Bilianskii (71) has started investigations on the role of microorganisms in the formation of enzymes in the therapeutic silts near Odessa. He found a

relationship between the number of microorganisms and the catalase activity of the silt and envisages the possibility of using the determination of catalase as a test for the characterization of the silts.

The reduction of sulfates in the lake Belovod' is practically entirely produced by microbes in the deposits on the bottom of the lake. Using labelled sulfate, Ivanov (72) has found that H_2S is produced at the rate of 0.12 mg./l. per day in deep places and 0.067 mg./l. near the shore.

An important review on the microbiology of the Caspian sea has been published by Kriss (73). It contains the following chapters: "The Role of Microorganisms in the Catabolism of Substances in the Caspian Sea;" "The Qualitative Distribution of Microorganisms;" "The Morphology of the Microbial Population;" "The Quantity of Microbes and Their Production;" "The Role of the Microorganisms in the Biological Productivity of the Caspian Sea."

This review includes much detailed information, figures and schemes, and also interesting considerations on the consequences of the modification of the flow of the river Volga (Volga-Don channel) on the microflora of this sea. The importance of river waters on the bacterial population of this sea and particularly in its shallow waters, as well as the presence of abundant zooplankton and phytoplankton which is favorable for the multiplication of microorganisms, has been stressed (74).

Infectious Diseases

The Vice-Minister of Health, Zhdanov, in a leading article which appeared in the No. 11 issue of the *Journal of Microbiology Epidemiology and Immunobiology* (*U.S.S.R.*), has reviewed the epidemiological situation in the U.S.S.R. and discussed the projects of combatting infectious diseases. Some statistical information cited in this article indicates that several diseases (plague, cholera, typhus, malaria) are either completely eradicated or nearly so. The auther emphasizes the procedures which should be used for controlling some other diseases, such as diphtheria, dysentery, brucellosis, etc. Elaboration of more potent vaccines and an increased effectiveness of vaccination procedures, as well as more research in epidemiology are envisaged.

This last problem, the theory of epidemiology, has been discussed in a special meeting of the Academy of Medical Sciences of U.S.S.R. The same journal, in its No. 10 issue, published an article by Agafonov, in which some general information on this meeting is given. It seems that no particular conclusions have been reached and various opinions on the biological and social aspects of epidemiology have been expressed. The role of immunological processes, of the variations or mutations of microorganisms, and of the influence of antibiotics on these transformations has been discussed. The problem of formation of new species and its importance for medical microbiology has been examined by Muromtsev (75), whereas the microbial variations and their influence on epidemiology and on the clinical aspects of infectious diseases have been discussed by Peretts (76). His opinion, based

mainly on personal observations with dysentery, but also on information taken from the literature, can be summarized in the following way: clinical observations show that in recent years several infectious diseases (typhus, scarlatina, dysentery) have become less severe. This is connected with the increase in the resistance of the hosts and with a diminution of the virulence of the microorganisms. Variations of microorganisms are provoked by modification of the environment, which normally is the host. When the resistance of the host increases, for example, by immunization during convalescence, the virulence of the infectious agent generally decreases (saprophytization). On the contrary, when a microbe is transferred to a very susceptible host, or to a host with diminished resistance (avitaminosis, etc.) its virulence may increase. Under the action of antibiotics, various modifications may appear, but generally the new strains tend to be less virulent, although their resistance to different physical, chemical, or biological agents may increase (76).

In a review on comparative pathology of infectious diseases, Sirotinin (77) assumes the existence of a relationship between the spreading of an infectious disease among different animal species and the period when the infectious agent first appeared. Thus, tuberculosis, which exists in a very large number of animal species, must be a very ancient disease, whereas dysentery which exists only in man although it can be reproduced to a certain degree in some mammals, must be of relatively recent origin.

At least 140 articles on epidemiology, pathogenesis, and diagnosis of infectious diseases provoked by microbes and viruses have been published and it would be impossible to mention all of them; only a few examples will be cited.

Studies on *C. diphtheriae* have shown that freshly isolated strains are frequently variants: some of those which decompose sucrose may be highly virulent and pathogenic, whereas strains having atypical morphological appearance or producing pigments are generally less toxigenic (78). The action of ultraviolet light (303 to 313 mμ) and of drying on various properties, including virulence, of the same microorganism has been investigated (79). Autolysates of this microbe increase lesions produced by the toxin or the culture; this activity is specific and is not related to the presence of a diffusion-increasing substance, which does not exist in all the strains, nor to a particular toxicity of the autolysate itself (80). The epidemiologic importance of "carriers" is stressed in several papers and different means of treatment and diagnosis have been proposed. Various methods for the determination of virulence and toxinogenicity have been compared and good results have been obtained using Ouchterlony's technique of specific precipitation in agar gels [see, for example (81)]. Use, not only of polyvalent but also of type-specific sera, is recommended for the serological differentiation of diphtheria and diphtheroid bacilli, some of their antigens being common to several types, but identification of variants or atypical strains could be achieved (82).

The hemolytic activity of various toxins has been studied, and it has

been observed that when two different hemolysins are mixed the final activity is higher than the sum of the two individual activities. This was the case when mixtures of *C. perfringens* and *C. oedematiens*, or *C. perfringens* and staphylococcal hemolysins were investigated (83, 84). The presence of hemolysins in extracts of organs infected with *C. oedematiens* or in exudates could be detected by a refined method in a relatively short time. The erythrocytes of infected animals, separated from the serum and suspended in saline, resisted lysis (85).

A series of three articles on the enterotoxin-producing strains of staphylococci and on their possible presence in alimentary products has been published in the No. 5 issue of the journal "*Voprosy Pitania.*"

The pathogenic activity of bacterial toxins on the nervous system will be mentioned in the last part of this review in the section concerned with the problem of the role of the nervous system in the pathogenesis of infectious diseases.

Use of kittens in experimental studies on *H. pertussis* has shown that they are particularly sensitive to the endotoxin and that their infection, by inhalation of microbial suspensions, provokes a short, local infectious process in the lungs. Sera obtained from hyperimmunized kittens possess high antitoxic titers but contains also antimicrobial antibodies. As many kittens are naturally infected with *Brucella bronchiceptica*, it is recommended that particular care be taken in the interpretation of results when these animals are used for experimentation (86).

Since the introduction of penicillin treatment cases of repeated scarlatina are frequently observed, which means that immunity is not acquired when such a treatment is applied. The necessity of finding a method to increase immunity is stressed (87). More than 50 per cent of children with rheumatic fever have in their history at least three infectious diseases, mainly angina and scarlatina. Living conditions (housing, humidity, absence of sunshine in the apartments, number, age and sex of other members of the family, etc.), the presence of hemolytic streptococci in the throat of other members of the family, are mentioned as favorable factors for "local family foci" in the epidemiology of this disease (88). The etiology and the pathogenesis of endocarditis has been discussed (89) as well as different aspects of angina. A series of articles on this last problem has been published in the No. 8 issue of the *Journal of Microbiology, Epidemiology and Immunobiology.* The etiology, epidemiology, and prophylaxis of these diseases, the different microbes found in the upper parts of the respiratory system, and the frequency of their presence in healthy persons or in carriers has been described. A small local epidemic of angina provoked by *Listerella* in man has been observed; the two strains isolated were of the serological type found in rodents, i.e., *Listerella monocytogenes*, type I (90). A survey of listerellosis in animals has shown that most domestic animals and poultry may be infected, but the more frequent infections are encountered in sheep. Fifty freshly isolated strains have been studied and compared to 25 strains from collections; no variants have been found (91).

Infectious diseases caused by bacteria of the intestinal group have been the subject of many articles. Epidemiology, control of carriers, diagnostic methods, etc., have been considered. Special "cabinets for infectious diseases of the intestine" have been created in some towns. Their role is to detect and control these diseases in order to prevent spreading of epidemics. Studies on the presence of *E. coli* and various *Salmonella* in town dust (92) and of the latter in rats have been performed (93). Different serological methods of diagnosis have been discussed, particularly those which use phage-typing of *Salmonella*. Among the publications concerned with the preparation, properties, and use of phages, a description of a new and simplified method for the detection of typhoid and dysentery bacteria with bacteriophages may be mentioned; its principle is based on the increase of the phage titer when phage is added to a culture where the corresponding bacteria are growing (94). The distribution of different phage-types of *S. typhosa* in different parts of U.S.S.R. has been studied by Zubkova (95); the stability of these types during several years (1944–1951) and the independence of biochemical or serological characteristics of a given strain from its phage-type has been stressed.

A hydrolysate of *S. paradysenteriae* has been used for an allergic diagnostic test. Preliminary results showed that a positive reaction appears in early stages of the infection and that this reaction persists during the whole period of the illness. The hydrolysate is not toxic and the reaction seems to be specific for dysentery. Rabbits can be used for studies on this allergic reaction (96).

Experimental dysentery infection and reinfection of monkeys has been used in studies on postinfectious immunity and on the problem of "carriers" (97). The postinfectious immunity is relatively short and unstable; the immunity induced by vaccination although of a low intensity shortens the period of persistence of living bacteria in the organism and of the excretion of them by the monkeys (98).

The question of excretion of dysentery bacteria by convalescents has been treated in several articles and it has been noted in many cases that it corresponds to a state of chronic disease; but some authors think that it may be due to reinfections by different types of dysentery bacteria (mainly of *S. paradysenteriae*). The infection of newborn children by their mothers has been observed and a bacteriological examination of pregnant women has been recommended.

The role of atypical strains in the epidemiology of dysentery has been envisaged and studies on such strains have been published by several authors. In most cases these strains belonged, by their biochemical characteristics, to the Flexner group, but in some cases also to other groups. Fisher *et al.* (99) have classified the atypical strains they isolated from convalescent persons and which have lost their characteristic agglutinability by specific sera into three groups: those which decompose rhamnose and dulcite, those which decompose only dulcite, and those which have no action on either sugar. The agglutinability of atypical strains could be re-estab-

lished, although only to a low titer, by cultivation on a glucose medium and this re-establishment was easier and more frequent with strains which had biochemical properties more similar to the classical Flexner type.

Histological examination of the intestinal walls in cases of acute dysentery in very young children has shown destructions of the nerve fibers, whereas in chronic cases different stages of dystrophy or destruction were observed (100).

An increased sensitivity to histamine has been observed in white mice, rats, and guinea pigs as a consequence of an injection of living suspensions of *Pastuerella pestis,* strain EV, or of autolysates of this microorganism. This state can be attained in a few minutes if a strong intoxication is provoked and can be temporarly inhibited by an antihistaminic drug (101).

Nikonov (102) has studied the distribution of *Vibrio comma* in the invaded organism and has found that this microbe multiplies abundantly in the gall bladder. He assumes that the bile system must be considered as a second focus of multiplication of the cholera vibrio and that this leads to an intoxication of the host. The lowering of bile secretion observed in early phases of the infection is considered by him as a negative factor and the prolonged presence of microbes in "carriers" is ascribed to their persistence in the bile system.

M. tuberculosis has been found in the bile of tuberculous patients and in dogs which received intravenous injections of suspensions of this microorganism. Since this penetration was observed after a period of time which is assumed to be insufficient to provoke a specific modification of the liver, the possibility of a passage of microbes from the blood into the bile is considered (103).

Several papers have been published on the epidemiology of brucellosis, on its prophylaxis, etc. The use of antiphage serum is recommended in order to facilitate the bacteriological diagnosis of *Brucella.* It results in more regular hemocultures and the growth of the first culture is enhanced on solid as well as in liquid media (104). Different preparations of allergenic extracts of *Brucella* have been compared and their practical use for diagnostic purpose has been discussed. Some of the described preparations showed sensitizing properties, whereas a hydrolysate which provoked no sensitization had a low allergenic activity (105). The hyaluronidase activity of *Brucella* and of the filtrate of their culture has been tested. Extracts and suspensions of cells have not showed such an activity, whereas lysates of cells and filtrates of culture were active. No strict parallelism was observed when hyaluronidase activity was compared to the capacity of increasing the diffusion of a color in rabbit's skin (106).

Epidemiology of tularemia and its occurrence in man have been the subject of publications in No. 2 and 9 issues of the *Journal of Microbiology, Epidemiology and Immunobiology.* The role of rodents, of sheep, and of ticks (*Haemophysalis otophila; Rhipicephalus rossicus*) in the transmission of this infection to man has been stressed and it has been recommended that

hunters and slaughter house workers be vaccinated. The acidity of normal human gastic juice is said to be able to destroy large numbers of microbial cells in a relatively short time (up to 2 millions in 30 minutes). Enzymatic activity was not involved in this destruction (107). Biochemical properties, antigenicity, and virulence of 46 strains of *Pasteurella tularensis* have been studied and it has been found that their antigenic specificity was identical, that virulence diminishes during storage at room temperature without loss of antigenicity, and that no one of these strains could decompose carbohydrates and higher alcohols (108).

Many publications on rickettsial diseases have appeared in the No. 7 and 11 issues of the *Journal of Microbiology, Epidemiology and Immunobiology;* most of them were concerned with epidemiology and clinical characteristics of typhus and Q-fever in different regions of the U.S.S.R. (Crimea, Azerbaidjan, Kasakhstan, Caucasus, Northern Asia, etc.). Transmission from animals is stressed and the presence of the infectious agent of Q-fever in ticks (*Hyalomna plumbeum* and *H. anatolicum Koch; Ixodes crenulatus, etc.*) living on different animals, has been observed.

The presence of rickettsiae in the kidney and their excretion in the urine have been studied on infected animals and it has been concluded that excretion is only possible when pathological modifications of the kidneys have been provoked by the infectious agents. The urine is not a favorable medium for the preservation of rickettsiae (109). The possibility of prolonged persistence of rickettsiae in man has been contemplated, but no definite conclusions have been reached on the possibility of latent forms (110). Series of experiments have been performed on the resistance of *Coxiella burneti* to various antiseptics, heat, ultraviolet light, etc. (111). The Weil-Felix reaction, agglutination, and the complement fixation tests have been used for the diagnosis of typhus with fresh and dried (on paper) sera; in most cases the agglutination and the complement fixation methods are recommended, the last one particularly, for examinations on dried sera, the agglutinins being said to disappear in 9 days when preserved in the dried state.

As has been mentioned previously, a new journal devoted to viruses appeared in 1956, but the present author had no occasion to consult it. This review is therefore incomplete on problems concerned with viruses. Nevertheless, many articles on these agents have been published in other periodicals, some of which will be mentioned. A new virus-induced disease was observed in 1948; it has been classified as an atypical form of meningoencephalitis transmitted by ticks (112). The clinical and diagnostic aspects of virus-induced neuro-infections has been studied by Filippovitch (113) who insists on the importance of laboratory investigations, i.e., isolation of the virus, transmission to animals, and detection of neutralizing antibodies.

In the first communication of an announced series of publications on the epidemiology of poliomyelitis, Solov'ev (114) discussed the epidemiological classification of this disease and the various possible mechanisms of its

transmission. Experimental results obtained in rats and monkeys infected by the oral route with poliomylitis virus confirm the observations that younger animals are particularly susceptible as compared with adults (115).

A study on postinfectious immunity in influenza has shown that antibodies begin to appear in the circulation on the third to fifth day of the illness, that their concentration is maximal on the twelfth to twentieth day, and that generally their titer is decreasing after one month. Differences between the antibody titers and their persistence have been noted for different types of viruses; thus, type B provoked higher titers, than types A or A_1. With these two latter types more cross-reactions have been observed. Persons who had severe forms of infection and who formed higher titers of antibody showed less susceptibility to reinfection than those who had suffered only a slight illness (116). Different preparations of dried influenza virus for hemagglutination and complement fixation tests have been compared and the best results and longer periods of preservation of their activity, have been observed with dialysates of allantoid-grown viruses; the method of obtaining of such preparations has been described (117).

In a series of publications a group of investigators describe the results of their work on measles. Typical symptoms, rise of temperature, appearance of skin eruptions, etc., have been observed in many of the 48 very young dogs infected with virus-containing material. The blood of these animals contained the virus from the second to the eighth day of infection, and could be used for passage to other animals. Formation of agglutinating (agglutination of virus-coated bacterial suspensions) and neutralizing antibodies could be established: these antibodies reacted with the virus isolated not only from dogs, but also from children and from monkeys (118). The virus has been cultivated in tissue culture (human embryonic lungs) and in some conditions 30 generations could be obtained, but already the fourteenth generation showed, by inoculation into monkeys, a diminution of its pathogenicity (119). Further studies with the virus cultivated on human embryonic lungs and embryonic skin confirmed that successive generations lose progressively first pathogenicity and then antigenic properties. Thus, after 6 days of culture, the virus isolated from a young dog, had provoked no clinical manifestations in a monkey, but protected it 5 weeks later against an inoculation of virulent human material. After 37 generations, the culture had lost its antigenicity (120). The virus, after passage through young dogs, has been also directly inoculated to monkeys and provoked only mild symptoms. These animals formed antibodies and were protected against an inoculation of virulent virus in human blood or washings of the throat which provoked measles in the control animals (121).

It has been proposed to use, for the diagnosis of infectious anemia in horses, the observation that the peripheral blood of infected animals contains macrophages which are transformed into siderocytes, by accumulation of hemosiderin (122).

Psittacosis was found in U.S.S.R. for the first time in 1951 on some imported birds, and the first human case was observed in 1953. In 1954, a

small epidemic outbreak (16 cases) occurred, the origin of which was found to be the zoo. Several strains of the virus have been studied using the agglutination reaction (123).

The possibility of multiplication of tobacco mosaic virus in bacterial tumors of tomatoes has been investigated. The inoculation has been carried out with a needle and, on the seventh or eighth day, virus could be detected in the tumor. The rates of multiplication of the virus in these tumors and in the leaves are analogous and the same titers have been observed in both cases. The penetration of the virus from the leaves into the tumor takes about two weeks, whereas its passage from the tumor into the leaves requires more than two months (124). A modified method for obtaining isolated strains of plant viruses, or for the separation of a mixture of viruses has been described (125).

Immunology, Vaccinations

In June, 1956, the thirteenth All-Union Congress of hygienists, epidemiologists, and microbiologists was held in Leningrad. It was attended by several thousand people and many hundreds of communications and conferences were read. From a short report on this Congress, which appeared in the last issue (No. 6) of the *Izvestiya Akademii Nauk, S.S.S.R.*, one can see that immunology and its practical applications have been largely represented. Studies on vaccination against brucellosis, tularemia, Q-fever, epidemic hepatitis, influenza, poliomyelitis, etc., are recommended by officials. Some more general aspects in immunology have been envisaged in conferences. Thus, Zil'ber discussed the role of the nervous system in immunity, the antigenic structure of some microorganisms and the "protective antigens" which can be isolated, the problem of "acquired tolerance," and also his personal results on antigens in cancer. Several persons treated the question of variation in microorganisms and its importance in prophylaxis. Berman and Slavskaia reported their results on the relation between age and immunological reactivity. Studies on the existence, during embryological development, of substances having different antigenic specificities, have been reviewed by Viazov, and Avetikian reported his results on the comparative antigenic stimulation in different animal orders. He found that only birds produce antibodies like the mammals; the lower orders are either "immunologically indifferent," as amphibians, or excrete foreign antigenic substances. Cytological studies on immunological processes have been accomplished by Pokrovskaia *et al.* and by Kokorin. The action of ionizing radiation on immunity and infections has been reported by Kiselev and by Troitskii, *et al.* The polyomyelitis vaccination problem has been discussed by several foreign guests.

Generalities; antibodies; serotherapy.—Phagocytosis of staphylococci in mice is greatly favored by penicillin treatment (126).

The capacity of an animal to form precipitins against a given antigen is said to depend not only on the individual characteristics of this animal but also on the species specificity of the antigen, and on the age and the sex of

the animal (127). Observed differences between the precipitin titers of immune sera from rabbits and their capacity to induce passive sensitization in guinea pigs are assumed to be due to differences between precipitins and anaphylaxis-provoking antibodies (128).

Studies on the species and organ-specificity of lens antigens during ontogenesis have been performed by Koniukhov. Using precipitating rabbit antisera and different embryonic organs of the duck, as well as lenses of ducks, of hens, and of frogs he found that during ontogenesis the antigens of the lens possess an organ-specificity and that it increases during development; a certain parallelism between the morphological modifications and the antigenic characteristics has been stressed (129).

Experimental endocarditis has been produced using a cytotoxic antiserum and the mechanism of its action has been discussed (130).

Several articles have been published on the preparation, purification, and therapeutical use of sera or γ-globulins from hyperimmunized animals (horses) or from human origin (blood or placenta). Good results have been observed in animal experimentation with γ-globulins, extracted from placenta by alcoholic fractionation, in prevention or in neutralization of dermonecrosis caused by *H. pertussis* in mice. The use of placental extracts possessing antipertussis agglutinins has been recommended (131). Favorable results were also obtained with γ-globulins extracted from horse antiplague, antiencephalitis (Japanese and tick-borne) and anti-*Brucella* (Vi-antigen) immune sera. The preparation of anticholera agglutinating horse sera has been described (132). Some useful modifications of the methods for the purificatoin of antitoxic horse sera have been found by experiment and an increase in the recovery of antibodies amounting to 5 to 7 per cent has been obtained (133). The presence and properties of proteases existing in antitoxic sera have been studied and their possible activity on antitoxins has been stressed; they are present in an inactive from, but are easily activated and their range of activity is relatively wide (pH = 5.5 to 8.0, with maximum at pH = 6.0) (134).

The methods of evaluation of the potency of antibacterial sera (antityphoid, antidysentery, antiplague) have been discussed in several publications, as well as the more general problem of statistical estimation of results obtained in the titration of biologically active substances. Differences in the therapeutic activity of antitoxins, dependent on the route of their injection, have been observed; intramuscular injections are said to be less effective than intrapleural or intraperitonial administrations (135).

Antigens; vaccinations.—Reports on vaccination against diphtheria with native toxoid and with purified adsorbed preparations indicate that better results have been obtained with the latter. Small increases in morbidity, mainly among children to the age of 3 years, are attributed to the incorrect application of the prophylactic agent and it is suggested that the revaccination schedule should be re-examined. Diminution of antidiphtherial immunity (136) among vaccinated children as a consequence of other infectious

diseases has been followed with the aid of the Shick test, and revaccination is recommended.

Active and passive immunization of guinea pigs showed that antimicrobial immunity, specific for *mitis* type, could be obtained in the absence of antitoxic antibodies (137). It has been found, again with guinea pigs, that injections of diphtheria toxoid provoke an increase in the phagocytosis of *C. diphtheriae* cells by the leucocytes of these animals and that this activity could be increased by repeated vaccination (138).

Studies on immunization against tetanus, or combined antidiphtheria and antitetanus vaccination have also been published and most of the results are comparable to those obtained with diphtheria. Adsorbed toxoid is recommended and experiments on volunteers or on animals enabled workers to establish the time of appearance of antitoxins in the circulation. In last year's review (186), some observations of Pletsityii *et al.* have been mentioned on the rapid appearance of resistance to toxins. The same author published new results (139) obtained on monkeys revaccinated with tetanus toxoid and claimed that 5 hr. after revaccination the titers of antitoxin increased $2\frac{1}{2}$ times and that 24 hr. later the titer was four times higher. But Kovtunovitch (140), repeating last year's experiments of this author, could not find that the classical "time factor" in the appearance of antibodies and of resistance to an injection of toxin is changed by the schedule used by Pletsityii (summation of partial excitations). Using mice and adsorbed toxoid, Markovitch & Vorob'ev (141) have tried to establish an equation expressing the response of these animals to a single injection of various doses of the antigen. This equation is as follows: $\log LD_{50} = a + K \log D$, where D is the injected dose and a and K are empirical coefficients, is said to correspond to experimental results obtained with doses varying from 0.005 to 1 ml. and the coefficient K is considered as being a possible means to express the quantitative aspect of the immunological reactiveness of animals.

Antigens of *H. pertussis* and vaccination against this microbe have been studied by several investigators. The antigenic and protective properties of different strains in various phases have been considered and it has been confirmed that phase I and virulent strains are to be used for the preparation of vaccine (142). The treatment of bacterial suspensions with ultrasound permitted obtaining of antigenic preparations which induced antibacterial and antitoxic immunity (143). A combined vaccine, containing microbial cells heated for 15 min. at 100°C. and a toxoid, has been used experimentally on animals and some volunteers; it did not provoke undesirable reactions and elicited good production of antibacterial antibodies; sera of immunized men protected mice against intranasal infection with living cultures (144). Another preparation, called "subneutral mixture" and consisting of whole antigens partially neutralized by specific antibodies, has also been tried in animals and complete immunity has been observed (145).

With Ouchterlony's double diffusion technique of specific precipitation in agar gels, which he has also used in studies on other antigenic mixtures,

Chistovich (146) has observed six different antigens in cultures of *H. pertussis;* various extracts have been compared for their antigenic components as well as for modifications of antigens during preservation of their solutions. At least 4 to 5 antigenic substances of this microbe are similar, if not identical, to those found in *Brucella bronchiseptica.* Studies on the pertussis hemagglutinin and the corresponding antibody in experimental animals and in children during infection and after recovery have been performed by Ivanova (147), but no definite conclusion could be reached on their protective action.

The problem of the antituberculosis vaccination with BCG has been discussed in the journal *Problemy Tuberkuloza* and the authors conclude generally that the techniques used are not yet completely satisfactory. The technique of vaccination called enterocutaneous is considered as being superior to the intracutaneous injection (148).

Chemical and antigenic properties of antigens isolated by Boivin's method from several strains of *V. comma* have been studied. The chemical constitution of these antigens was not modified by passage of the strains on animals (149). Vaccination methods and at least some of the vaccines used for antidysentery vaccination are considered by several authors as unsatisfactory; epidemiological surveys and experimentation on monkeys showed that prophylaxis was incomplete. Zak (150) found a direct relationship between the virulence of *S. paradysenteriae* strains and their capacity to elicit immunity, and proposed some definitions of strains which can be used for the preparation of vaccine. Various methods of extraction or fractionation of antigens from *Shigella* or *S. typhosa* and *S. paratyphosa* cultivated on synthetic media have been used and the immunogenic properties of these substances have been tested. The best results have been obtained with fractions isolated by ammonium sulfate precipitation of culture media (151, 152). Studies on the fate of typhoid antigens labelled with radioactive P injected or reinjected into mice are described by Ivanov *et al.* (153).

Batiuk found some formation of spores when *Bacillus anthracis* was cultivated at 43°C.; on the contrary, when the culture on a chicken broth was made at pH 7.1 to 7.2 and at 45°C., he obtained, regularly in seven days, from virulent strains a suspension of cells, possessing a low virulence and a high immunizing capacity; a single injection of such microbes was sufficient for immunization (154).

More than 20 articles have been published on the problem of vaccination against brucellosis and tularemia. It seems that the use of dried living microbes has produced good results. Technical details of the preparation of dried *Brucella* vaccines have been published by Vershilova *et al.* (155). The same author and others, in several articles, relate the results obtained in the vaccination of men (who professionally are in contact with possibly infected animals) and of sheep. The morbidity among the vaccinated was considerably reduced as compared to nonvaccinated persons, and also as compared to persons vaccinated with a heat-killed vaccine. Intravenous vaccine therapy in brucellosis has been investigated by Otraev (156). The introduc-

tion of dried living cells of *B. melitensis* into the conjunctiva of guinea pigs has induced immunity and the animals resisted 1 to 3 months later infection with a virulent culture. This route seemed to give a better results than the subcutaneous vaccination (157).

Combined vaccination of guinea pigs with living vaccines against tularemia and brucellosis has been studied and the relative proportions of the microorganisms in the mixture giving the best results has been determined (158). Several publications have been devoted to studies on the practical aspects of vaccination against tularemia, on the preparation of dried living vaccines, on the preservation time of active preparations, on the practical means of controlling their activity, etc. The spread of the microorganism in immunized and nonimmunized guinea pigs has been studied (159). Several articles have been published on the use of various preparations for allergic reactions in tularemia. It seems that a preparation called tularin gave very satisfactory results, and that its use in cutaneous tests should be preferred to intracutaneous injections, which may produce general reactions. Positive results were observed on a large number of vaccinated persons and on persons who had been infected (even 8 to 10 years before). [See, for example (160).] A polysaccharide-containing fraction has been isolated and tested for its allergenic properties. Sensitized rabbits gave positive reactions with 2 to 20 μg. of this substance, which also revealed itself to be devoid of toxicity and of antigenicity (161).

The problem of vaccination against Q-fever has been treated in several publications. Although immunologically vaccination gave positive results (complement fixation tests with the serum of vaccinated persons), the recommendation was made to continue research in order to diminish undesirable reactions which have been observed from its use (162). A vaccine prepared by formalin treatment of infected mice spleens showed the same immunogenic properties as the vaccine prepared on eggs (163).

Trachoma virus has been cultivated in the eyes and testes of rabbits and in the lungs of mice. Generally only 6 to 9 passages could be achieved. Different methods have been studied in order to obtain virus for the preparation of immune sera in rabbits employed in the diagnosis of trachoma using virus-coated microbes (164).

Cancer.—As in last year's review, some publications on cancer are included in this article. They are mentioned here, under the general heading of immunology, because immunological methods have been applied in the works cited.

On the occasion of the sixtieth anniversary of Prof. L. A. Zil'ber, his collaborators and colleagues have published a book titled, "*The Problems of Pathogenesis and Immunology of Tumors*,"[3] which contains some 30 articles on these problems. It would be impossible to summarize them here; only a few of them will be cited. Among the articles on tumor antigens, several are concerned with virus-induced tumors such as the rabbit papilloma, the

[3] Prof. G. V. Vygodchikov, Editor, Moscow, U.S.S.R., 1956, MEDGIZ.

"milk factor" of mice, the chicken sarcoma, etc.; others with the specific antigens found in human and animal tumors. As in previous publications, anaphylactic reactions in sensitized guinea pigs are used as a general immunological method, and desensitization with the corresponding "normal" tissue antigens is employed in order to prove the specificity of antigens extracted from tumors. In some cases specific precipitations have also been used, for example, in the study of specific antigens in mice hepatoma elicited by cancerogenous substances. These antigens were found in the period which precedes the growth of the tumor (Korosteleva). A method for the fractionation of tumor or organ extracts, as well as a special centrifuge and some results of electrophoretic analysis of the fractions have been described (Abelev *et al.*). Ten articles concern the problem of antibodies, the use of complement fixation reactions in studies on tumors, immunization against the Brown-Pierce carcinoma, etc. Photomicrographs (electron microscope) of some purified preparations extracted from spleens of human leucosis cases and obtained by the same methods as used in the purification of viruses, are reproduced. They show abundant bodies (absent in analogous extracts from normal human spleens) having a diameter of 100 to 140 mμ (Parnes).

A new method for the extraction of an antigen from tumor tissue has been described. It consists of using formalin-treated tumors. Normal tissues, in these conditions, contain no comparable antigenic substances. The somewhat purified substance extracted from formalin-treated tumors is described as a complex; the amino acid content of the protein component in this complex has been determined (165). Extracts from organs and from the urine of leukemic patients are said to contain specific substances which are antigenic and elicite anaphylactic shock and precipitation with corresponding immune sera, previously absorbed with analogous extracts from normal organs (166). Rabbit immune sera have been used in studies on the growth of the Brown-Pierce carcinoma in tissue culture, but the presence of antibodies reacting with normal tissues limited interest in the obtained results (167).

The Role of the Nervous System in the Pathogenesis of Infectious Diseases and in Immunology

Research on problems connected with the physiology or pathology of the nervous system continues to be one of the main trends in biological investigation in the U.S.S.R. Therefore, publications reporting experimental work on the role of this system in infections or in immunological processes have been grouped in this separate section. Two different aspects of these problems will be distinguished: the action of microorganisms or of their toxins on the nervous system, and the intervention of the central nervous system in infectious diseases and in immunization.

The mechanisms of the action of botulinus toxin on respiration and on the nervous center regulating it (168), and on the reflector activity of the spinal cord (169) have been investigated. Differences in sensitivity to the typhoid toxin of sympathetic and para-sympathetic inervations of the heart have been described (170). The phenomenon of blowing up of the lungs in rabbits receiving intravenous injections of the dysentery "complete antigen"

is assumed to be induced by lesions of the superior sympathetic nodes (171). The conditioned reflexes of rats have been influenced by injections of tetanus toxoid but, after repeated injections, these reflexes were more easily re-established (172).

The capacity to respond to an injection of antigens in schizophrenic patients has been investigated and a certain parallelism has been found between the severity of the case and the diminution of the response (173). Studies on the modifications of the central nervous system and particularly on the conditioned reflexes, in tuberculosis and in brucellosis, have been performed and it has been concluded that the state of an infected animal and the final issue of an infection depend on the functional state of the central nervous system [see, for example (174)].

The possibility of the participation of reflex mechanisms in immunological processes is still a subject of lively discussions and of experimentation. Some authors, for example Krylov (175), persist in attributing to the reflexes the main role in antibody response, and try to explain the experimental results obtained by other investigators on the basis of their theory. But many others, although admitting that the quantitative aspect of the response may be influenced by the central nervous system, insist on the importance of direct contacts of the antigen with the cells of the reticuloendothelial system and admit that the specificity of the response is independent of the central nervous system [see, for example (176)].

An increase in antibody titers under the influence of an excitation of the brain cortex has been found by several investigators in rats and rabbits immunized with various antigens; such experiments were performed also on rabbits whose cerebellum has been extirpated (177, 178, 179, 180). Some authors, in the discussions of their experimental results, admit that they could not prove the existence of a specific effect of reflex stimulations (181, 182). The influence of the central nervous system on streptococcal infection in guinea pigs (183) and on gas gangrene in operated rats has been studied; in this last case the observed modifications are attributed to the operative trauma and not to the extirpation of a certain part of the brain (184).

Many investigators in their studies of the role of the nervous system in infectious diseases and in immunological processes have utilized pharmacological products. Medicamental sleep is said to provoke some alterations in the immunological response of experimentally infected animals (formation of antibodies) and in the infectious process [See, for example (185)]. It has been assumed that the effect of diphtheria toxin on animals under the narcotic action of ether has been lowered (186), as well as after the injection of caffeine in young dogs (187). Regular increase in the precipitin titers of immunized animals has been obtained by subcutaneous injections of caffeine (188). But in experimental immunization of mice with dysentery vaccine, it was found that this drug provoked a negative result, whereas bromide increased the immunological response (189). Stimulation of the formation of antibodies by bromide has also been observed in rabbits (190). The allergic reactions in animals immunized with living *Brucella* were somewhat delayed and were milder when bromide was administered, whereas under the action

of caffeine the reactions were accelerated and stronger (191). In rabbits, the local skin reaction on the injection of the "complete antigen" extracted from dysentery bacteria, has been studied using local and general anesthesia; diminution or disappearance of the reaction in these conditions have been reported (192). In most of these publications the experimental results are interpreted as showing the important role played by the central nervous system.

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PREDICTION OF PLANT DISEASE EPIDEMICS[1]

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Introduction

During the past decade or so we have been finding that we can not control certain diseases properly without some prior indication as to how they are going to behave in the coming period; that is to say, without predicting where and when they will occur and taking action according to the prediction. This is true in spite of all the recent advances in chemical control measures. Unless we can know just when sprays or dusts, however effective they may be in themselves, should be applied we are apt either to use them extravagantly to take care of the disease if it should appear, or not at all on the chance that it will not appear. Either alternative is wasteful. In the first case the chemicals often will not be needed; in the second case the chance may go against us and the crop be lost. For some diseases no effective or practicable chemical control measures are available so far. Nevertheless, forecasting gives the grower time to revise his plans in order to avoid or reduce losses. Wheat leaf rust is an example of this type.

Forecasting the incidence of a plant disease must take into account not only the disease itself but the weather also. It has been said that this might seem to be a practically impossible combination; on the contrary, the very fact that the development of a disease depends on some particular factor or combination of factors which we are able to determine and prepare for, is our only basis for prediction.

Experience shows that it is not necessary to know exactly what the circumstances will be in order to make a very satisfactory prediction. We can say "*If* the weather is this, *then* the disease will be that," and even a conditional prediction can be most useful. The minimum requirement is some observed sequence of conditions and disease. The explanation, if we are able to find it, may be simple or complicated but in the meantime the sequence furnishes a starting point for prediction.

Naturally, the reliability of forecasting is greatly improved if the reasons why the disease develops under some conditions and not under others are known. Experimental investigation is necessary to show exactly at what stage in the development of a disease any single factor is decisive; that is, what the critical points are, and how and to what extent the influence of any one condition is modified by different combinations. Experience with late blight, for example, shows definitely that the relative importance of each separate weather element varies with the intensity and fluctuation of other conditions.

In the following account it will be seen that forecasting is not a new development in plant disease control, that it is becoming widely used with

[1] The survey of the literature pertaining to this review was completed in December, 1956.

increasing success, and that much of its success is due to the continued applications of results from research.

Potato and Tomato Late Blight Outside the United States

Holland.—In western Europe the Netherlands pioneered in the development of forecasting and spray warning services for the control of the late blight fungus. Thirty years ago Van Everdingen (187) analyzed the combined effect of several weather conditions on the development of *Phytophthora infestans* (Mont.) D By. and evolved four rules, known as the "Dutch rules," upon which the appearance of blight was observed to hinge. They are: (*a*) dew during at least 4 hr. at night, i.e., night temperature below the dew-point for this length of time; (*b*) a minimum temperature of 10°C. (50°F.) or above; (*c*) a mean cloudiness on the next day of 0.8 or more; (*d*) measurable rainfall during the next 24 hr., at least 0.1 mm. When all four conditions obtained in Holland, control measures were recommended.

A survey made in Holland in 1926 proved the validity of these Dutch rules. Based on them, a warning service was set up which was carried out for the next 24 years (156, 157). The Royal Dutch Meteorological Institute at de Bilt installed several special stations (156, 157) with weather equipment to afford records of dry, wet bulb, and minimum temperature data, amount of cloudiness at 8 hr., 14 hr., and 19 hr. with rainfall being measured at 8 hr. All these data are taken at a height of 40 cm. above ground level and, if possible, in, or near the vicinity of, a potato field. The stations send messages by telephone or telegraph every morning when the weather fulfills the conditions of dewpoint and minimum temperature. During the following day observations on cloudiness and rainfall are added. When conditions are right for an outbreak of potato blight, the warning service issues blight warnings by radio. Very satisfactory results have been obtained from this "cautionary service" (156, 157, 188 to 195).

In 1948 and 1949 a method was sought (156, 157) which would make it possible to give warnings without using the special stations, for it was shown that in many cases the stations gave only a local and not a regional warning. Also, farmers asked the Institute to predict a critical period for the outbreak of potato blight. Although it was possible (11) to provide reliable warnings based on relative humidity and minimum temperature observations at the 40 cm. level, normal synoptic weather observations from the regular weather stations were employed to give predictions of blight outbreaks. This latter method has two great advantages: the first is that all observations needed can be taken from the weather maps and from the synoptic stations which send their observations to the Meteorological Institute every hour; and, secondly, from the farmers' point of view this method of warning is a regional warning. In some cases it is possible to predict a period in which the weather will fulfill the conditions of temperature and relative humidity so that the farmers have one or two days time to spray their potato crop before the predicted outbreak of blight. In many cases where critical periods were predicted

locally, the prediction was followed by some rainy days when spraying, of course, was not effective.

The criteria for such predictions are the result of a comparison of a great number of observations from 2.20 m. and 40 cm. levels. These criteria are: (a) two consecutive days with a mean relative humidity of 82 per cent or more (further investigation showed that potato blight also broke out when in one of the two days the mean relative humidity descended to 79 per cent), the mean being taken from observations at 8, 14, and 19 hr.; and (*b*) for one of the days, at least, the minimum temperature must be 10°C. or more.

England.—During the past quarter century approximately, English workers have tested experimentally the Dutch rules for late blight forecasting (9, 10, 11). Under weather conditions prevailing in England the rules were generally satisfactory although the blight did not appear as quickly as the rules predicted. In England dew formation and periods of high humidity with sufficiently high temperature, minimum temperature 50°F. or over, are looked upon as insuring development of blight (12 to 16, 214). Beaumont & Hodson (13) added a fifth rule to those formulated by the Dutch to apply under conditions in England, particularly in Devon and Cornwall. The additional condition, or fifth rule, called for periods of not less than two days in which the relative humidity (at 3 p.m.) was higher than 75 per cent. Even when air temperatures did not reach 50°F. in the West Cornwall region, blight occurred after periods of high humidity (15), the cumulative effects of high humidity compensating for lower temperatures. According to Beaumont & Staniland (*loc. cit.*), the humidity figure is a much more definite boundary as it marks the line between spore production and absence of spore formation.

In 1937 Beaumont & Staniland [(16) 1937 report] reduced these five rules to two, since equally good results were obtained from their use, viz., (*a*) minimum temperature 50°F. or over; (*b*) relative humidity not under 75 per cent for at least two days. This modification of the Dutch rules was adopted for future use (*loc. cit.*) and became the basis of experimental forecasting of blight conditions in England (11). Although the Dutch rules applied to southwest England to a certain extent, they tended to forecast blight appearance earlier than eventually happened (11).

Continuing the work in this decade, the results of the first six years of an investigation into the possibility of forecasting outbreaks of potato blight by the Beaumont rule throughout England and Wales are given in reports compiled by Large (104, 106). The results indicate that broad regional forecasts of a blight outbreak date could be made successfully for all parts of England and Wales based upon the identification of Beaumont weather periods from reports furnished by a cooperating network of synoptic weather stations. The system is designated as an "operations chart" method. In making predictions, indication of blight weather periods must be taken into account from the entire network and allowances made for seasonal and regional differences in the forwardness of the crops. The "zero date" before

which Beaumont periods would not be followed by general outbreaks on main crops is about the last week in June except in southwestern England, western coastal areas, and around the Wash. Blight assessment on the haulm at the 0.1 per cent stage of the British Mycological Society's key (134) in a potato field was taken as providing an "outbreak" record. This stage is usually reached after the fungus has passed through two or three generations. Premature warnings would result if an earlier stage were used. From the curves of blight progress obtained, there appears to be no constant relation between the date of blight outbreak and the date on which rapid destruction of the haulm begins.

These investigations are to be continued and provide valuable information for estimating blight losses and also correct advices on spraying.

Scotland.—Grainger's experiments (70) in Scotland confirmed Beaumont's (11) general theories on the temperature-humidity rule. Forecasts were all valid after the last days of June for the years 1944 to 1948. Since they were not valid before the end of June, Grainger specified that time as the "zero time" of validity for the forecasts in southwestern Scotland. Foister (65) confirms that for the west and southwest of Scotland Beaumont's temperature and humidity rule is valid, to a very large extent, for a crop in the neighborhood of the reporting service. In the east and north areas consistent data have not been obtained, the data being "marred" by the appearance of blight within a very few to ten days after the forecast instead of after the longer period experienced by Beaumont (11). In these areas forecasting seems to be almost a problem of locality. The reason is that crop environment, combined with relatively large topographical variations even within one area, tend to give individuality of disease conditions to each field, and individual forecasts, of course, are beyond the scope of a forecasting scheme.

Successful forecasting of the first appearance of blight and of weather suitable for subsequent attacks would be invaluable for timing protective sprays to diminish spread of blight to tubers and would reduce the level of tuber decay in most years. Similarly, forecasting the first appearance of blight in the Ayrshire district of Scotland where potatoes are grown for seed, would greatly reduce loss as blight usually appears when the potatoes have reached the period of effective growth. However, seed growers are not disturbed by the fact that blight may kill the haulms completely towards the end of the growing season, thus acting as a cheap and natural alternative to chemical haulm destruction (65). Also, Van Everdingen's (187) and Cook's (50 to 54) criteria on rainfall in relation to haulm blight forecasting would not hold directly under Scottish conditions, since spraying is uneconomical because haulm blight usually occurs too late to affect the yield. The forecast of heavy or continuous rain might be helpful by allowing growers to burn off the tops before the rain encouraged extensive tuber infection. A destructive spray, however, applied just before blight appeared, would prevent establishment of blight on foliage at lifting time, impede heavy infection of tubers and loss in storage, and would allow normal digging procedures

to go on unhampered. This spray would be very effective, particularly when there is sufficient rain in late season to wash the spores down to the tubers.

Recently, Grainger (71, 72) has designed a self-calculating blight forecast recorder, called the Auchincruive Potato Blight Forecast Recorder, for use in determining critical periods for blight occurrence. This machine, whose recording elements are bimetallic coils, is inexpensive, accurate, and easily interpreted without any special skill. Record of the relative humidity is made by a "calculator" pen, calibrated to give a line coinciding with the temperature line, also recorded graphically, at a relative humidity of 75 per cent. With the development of such instruments as the Auchincruive Recorder, the accuracy of blight occurrence forecasts should be markedly increased.

Ireland.—In recent years in Ireland (27) a new set of criteria has been formulated as a result of controlled laboratory experiments on the environment favorable to the development of late blight. Using standard hourly meteorological reports, the conditions for blight to develop in Ireland are: (*a*) A "humid period" of at least 12 hr. with the temperature equal to or greater than 50°F. and the calculated relative humidity equal to or greater than 90 per cent. (*b*) Free moisture on the leaves for a subsequent period of at least 4 hr. or, without precipitation, the alternative requirement is a further 4 hr. beyond the initial 12 with relative humidity at least 90 per cent, i.e., the presence of condensed moisture on the leaves for at least 4 hr. after sporangial formation. These conditions specify minimum conditions preceding the first appearance of the disease in any year. Bourke (*loc. cit.*) considers that later it might be found necessary to set up two models, one for the earliest attacks and another more exact one for forecasting important changes in the intensification of the disease later in the season.

In Ireland use is made of existing hourly weather reports from ordinary weather stations of the synoptic network rather than dependence upon special stations in the neighborhood of potato fields, as in The Netherlands. Although this system does not account for any local effects of the disease, it does afford bases for indicating the general progress of the disease which is of main importance. Four kinds of weather categories are found to favor blight in Ireland: (*a*) broad warm sectors, particularly with further wave development following; (*b*) depression or fronts or both (including thundery lows or troughs), quasi-stationary over the country, and giving lengthy periods of damp, cloudy weather; (*c*) westerly sequence of weather, with quick succession of frontal troughs, uninterrupted by direct cold air outbreaks from the Arctic, so that overcast weather is virtually continuous and rainy spells are frequent; (*d*) persistent fog and, in particular, inland penetrations of wet sea fog. Owing to the incubation period of the disease, and the lapse of a few days before visible evidence of the fungus is seen, it is possible to attempt forecasting blight weather before it occurs.

Bourke (28) in his next technical note reviews the organization of the blight weather warning service and the whole blight situation in Ireland in 1952. It is an assessment of the accuracy of the blight warnings in that year

and the validity of the "Irish Rules" on which they were founded. The issuance of warnings in timely relation to spraying operations was satisfactorily carried out. The results showed that no major modification of the Rules would be necessary as the occurrence of blight weather, as defined in the Rules, proved to be in agreement with the course of the disease in Ireland as a whole, as well as in the districts for which reports were available. The inhibiting effect of a drought in the 1952 season was noted. The year's work also showed that blight weather spells are to be regarded of lesser importance unless they attain an effective duration of 10 hr. or more. Analysis also confirmed the importance of moisture on the foliage and where doubt existed on the likelihood of the foliage being wetted, it was safer to assume that conditions were not in agreement with the minimum criteria of the Irish rules.

In comparison of the Beaumont (English) rules with the Irish rules, it was found that although both systems indicated the dates of first important attacks simultaneously, the English rules tended to exaggerate the danger of intensification of the disease from mid-July onward. Under conditions in Ireland it appears that the Irish rules, which represent minimum criteria and which break up favorable weather into shorter and more measurable lengths, are better adapted to bringing to view the significant weather spells and to developing a technique for forecasting probable events. The "Irish Rules" consider every spell of weather which could aid growth of the fungus.

The experience gained in the 1953 potato-growing season in Ireland is reported by Bourke (29). Lengthier periods of blight weather, at least 10 hr. long, are more efficient in spreading or intensifying the disease. In the spread of blight, also, separate 10-hr. periods have more effect than a single effective period of 20 hr., particularly if one or more spore generations intervene.

Finland.—Rainio (159) reports that difficulties involved in timing application of sprays can be overcome by provision of an adequate number of observers and by preparation of special isophane maps indicating the relationship between climatic factors and the development of blight on a given variety at a certain time in different parts of the country.

France.—The vine mildew observer-type system, including issuance of spray warnings, was utilized also in forecasting late blight development in France. In 1932 Dufrenoy (62, 160) proposed four conditions favoring the infection of potato plants. They were similar to those of the Dutch rules and were conditions generally favoring germination of zoospores. These conditions are: (*a*) during the night the temperature must not go lower than 10°C. (optimum temperature for germination of zoospores between 10° to 13°); (*b*) the leaves must remain covered with dew at least 4 hr. of the day following the night dew; (*c*) the sky must remain cloudy, i.e., 0.8 of the sky should be covered with clouds; (*d*) there must fall at least 0.1 mm. of water in the form of rain.

Viennot-Bourgin's data (197) showed, however, that under exceptional climatic conditions and even in the absence of Van Everdingen's requirements for late blight (*loc. cit.*), serious attack may take place at an advanced

stage of growth when infection tends to become rapidly localized in the ripening tubers. Limasset (108) found that blight continued to spread, even when humidity was under 75 per cent and Beaumont's two rules were not satisfied.

Recently, as reported by the Working Group on "Weather and Plant Pathology Problems of the Commission for Agricultural Meteorology of the World Meteorological Organization (30), the favorable meteorological conditions for attacks of potato blight in France are essentially the same as those set forth by Dufrenoy (*loc. cit.*) except for the following: (*a*) a lowering of the temperature which reduces the plant to a state of receptivity; (*b*) a warm period (18° to 21°C.), favorable to the development of the fungus in the tissues of the potato plant after the temperature-moisture conditions suggested by Dufrenoy have been met. The meteorological limits, however, are not rigidly defined. Continuing work is done each year to adjust the rules to their true values.

Germany.—The German contribution to late blight forecasting in the early period of the past quarter century was in basic studies of the effect of environment on the blight fungus. Müller (136) points out that the intensity and extent of an epidemic are influenced by the weather conditions before attack, not while the disease is actually in process. The spread of the fungus, however, is governed by the reaction of the host as well as by meteorological conditions. Fluctuations in relative atmospheric humidity (148) appear to be more detrimental to sporangial development than changes of temperature within a fairly wide range (2° to 34°C.).

In 1953 Johannes (97) undertook, at the Brunswick branch of the German Biological Institute, a fundamental study of the problem of forecasting potato blight outbreaks. His study is based on the assumption that microclimatic factors are primarily responsible for the development and epidemic occurrence of the pathogen. In his 1951 studies, confirmed in 1952, he found that two periods of high relative humidity, at least above 82 per cent for more than $4\frac{1}{2}$ hr. had to prevail in potato stands for at least 33 hr. to ensure development of an outbreak. Mass production of the sporangia of *P. infestans* could be interrupted by a fall in relative humidity to 72 per cent for as short a period as $2\frac{1}{2}$ hr. during the critical time for sporangial development.

Voekel (199) at the same Institute made a critical analysis of the potato blight notifications submitted to the Biological Institute between 1925 and 1944 in connection with the preparation of an atlas of "regions of damage" areas in Germany. "Regions of damage" are defined as areas in which a plant disease or pest is endemic. By combining records from districts supplying the largest number of epidemic reports with those in which blight assumed virulent form in five or more years, a conspectus of the individual "regions of damage" in different provinces is secured. However, these studies afforded no definite correlation between environmental factors and blight development.

Thran (177) describes two sets of criteria which were applied in the province of Schleswig-Holstein. Adding up the mean daily temperatures for

the 1st of April, he calculates that the zero dates for outbreaks of blight on two potato varieties [Erstling (Duke of York) and Sieglinde] will be reached at totals of 800°C. and 1100°C., respectively. The second set of criteria defines a critical period, viz., a maximum temperature above 23°C. for two consecutive days, with a minimum not below 10°C., the mean daily vapor pressure over 12 mm., and the mean amount of cloud not below 0.5. The incubation period ranges from 2 to 3 days with a mean temperature of 20°C. to over 13 days at 12°C.

In the potato-growing zone of north Hanover Kabiersch (99) reports the application of Post & Richel's rules (157). However, a number of critical periods from mid-July did not result in outbreaks of blight within the specified time of 15 days.

Uhlig (179) made a critical survey of the existing methods for the prediction of potato blight outbreaks. These included the Dutch, English, Irish, and German studies (all treated herein). From his survey he concluded that, although there might be agreement for the whole of central Europe on the meteorological factors necessary to define critical periods, crucial threshold values will differ in every region. This fact has been borne out by the reports of several workers, particularly Hyre and Wallin in America, Grainger in Scotland, and Bourke in Ireland.

In evaluating critical weather periods early in the growing season, Uhlig (180) stresses the importance of phenological observations on degree of culm closure to provide proper interpretation of meteorological data. He also points out that *Phytophthora* evolves slowly at first from small, primary foci of infection but that each succeeding generation develops more rapidly. The curve of development evolves in a step-like fashion, with ever broader steps following. It appears that three, or at the most five, generations of fungus spores should be noticed before spraying recommendations are made. Although spraying does not prevent the rise of the infection the degree of rise is substantially lessened. Current reporting on critical periods following the first warning provides assistance in determing the spraying procedure.

Greece.—Van Everdingen's rules (107) were found to hold in Greece during a severe epidemic of potato blight which occurred near Chalcis on Euboea Island (223). In the twenty-day period when the disease was severe in 1949, weather conditions favorable for disease development prevailed on four days, with rainfall on three other days contributing to disease extension.

Norway.—During World War II a warning service was established for late blight of potatoes, using the English modifications of the Dutch rules (98). The method was employed from 1941 to 1947 but met with small success, owing to the varying climatic conditions and marked orographic weather effects in the mountainous country of Norway.

Russia.—Russian workers followed Van Everdingen's rules (107) in forecasting late blight development (141). The length of the incubation period of the disease was found to be dependent mainly on minimum, mean, and maximum temperatures prevailing on three days following the "critical" day, with relative humidity not affecting the length of this period appreci-

ably. From a statistical analysis of the problem a "nomogram" was constructed by which the time of appearance of late blight could be determined from these data.

The only recent note on this subject in the literature available to us here is that of Schuster (170) describing the organization of the Russian Plant Protection Service, one of whose functions is the conducting of a large-scale potato blight warning service.

Brazil.—We have been unable to find anything in the literature on plant disease forecasting in Brazil. However, Bitancourt (18) reported that A. C. Andrade of the Institute (São Paulo Instituto Biologico) had analyzed the meteorological records of several localities in the State of São Paulo for the previous 14 years and found that a critical period of mean temperature below 21°C. and rainfall above 30 mm. for a one-week period occurred a few weeks before the appearance of tomato late blight, as recorded in the Institute's files. This has taken place five times since 1937. Critical periods occurred only twice without a corresponding record of late blight. No records of the disease exist when the critical period did not occur. Andrade concluded that prediction of an epidemic can be made on the basis of the occurrence of two consecutive weeks or four alternate weeks of low temperature and high rainfall in the State of São Paulo. It was planned to make announcements on spraying whenever these weather conditions endure for a whole week.

Peru.—In Peru (61) studies were undertaken on the meteorological conditions influencing the incidence and development of *P. infestans* on potato with the purpose of establishing a service for the prediction of and prevention of diseases and pests of cultivated plants.

Following an extremely virulent attack of *P. infestans* on potato in 1947, growers were forced to employ various fungicides in subsequent years. Under Lima conditions, the factors for initiation of late blight on potato comply with those established by Beaumont and Staniland (*loc. cit.*, late blight forecasting in England). In 1945, a year without blight in Peru, it was noted that the relative humidity was almost permanently below 95 per cent and that very few days had nocturnal hours with 96 per cent. In April and May the humidity averaged less than 75 per cent, rising and falling in the months of June, July, August, September, and the first 15 days of October, the average being maintained near 75 per cent with few exceptions. In the last half of October the average humidity was below 75 per cent. In contrast, in 1949, a year of intense infection, the relative humidity was 100 per cent during many hours on all nights. In 1947 and 1948, also blight years, the nightly relative humidity was between 98 and 100 per cent. Since in both blight and nonblight years the relative humidity for the majority of days was on the average near 75 per cent, the daily nocturnal relative humidity is held to be more important under Lima conditions. With nightly humidity of less than 95 per cent there is no danger of blight infection. With relative humidity ranging above 95 per cent, the possibility for an epidemic attack is indicated. In other words, a nocturnal relative humidity of less than 95 per cent with considerable variation in daily temperature produces conditions unfavorable

for blight. In contrast, high nocturnal humidity, with little variation in daily temperature and with temperature minima between 10° and 13°C., provokes a heavy attack of *P. infestans*.

Australia.—While there is no formal plant disease forecasting scheme in operation in Australia (115), warnings are issued for late blight of potatoes and tomatoes when seasonal conditions are favorable for development of the disease. These warnings are issued by regional agronomists and other officers through the country press or via the radio on agricultural programs.

Japan.—Considerable attention is being given to the study of late blight of potato in Japan (165). Appearance of the disease and its progress northward are noted in various parts of Japan, with appearance serving as warning of possible damage to the north. However, the correlation is generally not sufficiently good to serve as a basis for prediction except in Shizuoka Prefecture, where appearance of the disease is a reasonably reliable index of occurrence some three weeks later in the more northerly and generally higher prefecture of Nagano.

Potato and Tomato Late Blight in the United States

The need for a warning service for potato and tomato late blight in the United States was evident long before a cooperative forecasting project was established. Moreover, a considerable amount of experience by various groups already had shown that appearance and spread of late blight and some related fungi could be successfully anticipated, provided only that means for watching and reporting progress were available.

The event that finally brought about the establishment of the regional Forecasting Project was the severe, widespread, and unexpected epidemic of tomato late blight in 1946. The Project was organized to include a warning service and contributory research for the three downy mildew diseases, late blight of potato and tomato, blue mold (*Peronospora tabacina* Adam) of tobacco, and downy mildew [*Pseudoperonospora cubensis* (Berk. & Curt.) Rostow]. of cucurbits. In North America the seasonal progression of these crops from south to north all the way into Canada is particularly well-adapted to observation and prediction on a widespread regional basis. Canadian pathologists cooperate in the warning service.

Appearance and spread of tomato and potato late blight, blue mold, and downy mildew of cucurbits are reported promptly to the Plant Disease Epidemics unit at Beltsville, Maryland. The reports are compiled into a warning letter sent twice weekly during the growing season to the pathologists in the various States and Canadian provinces and other authorized recipients. By watching occurrence and severity in areas with an earlier growing season and applying their own previous experience, together with the weather forecasts, the key pathologists are able to judge rather accurately whether or not the local crops are likely to be affected and, if so, how soon and how seriously and can make their forecasts accordingly. Manufacturers of control chemicals and equipment also use the warning letters as a guide to allocation of their products. Detailed accounts of the organization

and progress of the warning service for the past nine years have been published (123 to 125, 127 to 131) and recorded by mimeographed letter reports for 1953, 1954, and 1955.

The late blight research program was divided among three different regions of the United States, north-central, northeastern, and southeastern. The program has four main objectives: to develop workable prediction formulas for regional forecasts; to investigate the effect of environment, especially microclimate, on the establishment and survival of the pathogen; to determine the existence and distribution of strains of the fungus, some perhaps more virulent than others; and to study the life history of the fungus, with special attention to factors influencing germination and infection. The results obtained during almost ten years of research have contributed a great deal to fundamental knowledge of the organism and the factors that affect development of blight, and have laid the groundwork for some successful experiments in forecasting occurrence on a regional basis.

Forecasting methods.—Environmental relations have been explored from several angles in each of the three major regions. One approach has been an analysis of weather data and potato late blight records for the past half century or more, or sometimes for shorter periods but as long as possible. This has been accomplished by several workers in the Crop Plant Disease Forecasting Project and also by H. T. Cook.

In the Atlantic Coast region, both north and south, some very satisfactory schemes for predicting the occurrence of late blight have resulted from these comparisons. They depend on observed association of late blight appearance and spread in combination with temperatures within a definite range and with rainfall above a certain minimum. These predisposing weather factors must exist together for a period of time long enough to allow infection of the host and development of the fungus. In practice the current season's weather data are compared with the standards favorable for late blight occurrence derived from these long-time records.

In the southern Atlantic Coast region, after the severe blight epiphytotic of 1946, Cook (50, 51) studied the relation of temperature and rainfall during the preceding 17 years to blight on potatoes and tomatoes in eastern Virginia. It was found that the critical period for blight development in that area started on May 8. The cumulative mean rainfall line, starting with May 8, was plotted for blight and nonblight years and a median line was designated the critical rainfall line. The critical temperature was taken at 75°F. Severe epidemics were likely to follow a two-week period when rainfall was continuously above the critical rainfall line and the temperature below 75°F. Cook (54) later extended the analysis to 31 years (1917 to 1947) at Norfolk, Virginia, and Charleston, South Carolina. He concluded that forecasting blight on this basis during that period would have been 84 per cent correct for the Norfolk area and 81 per cent accurate for the Charleston area and would have made it possible to eliminate unnecessary spraying in 77 and 58 per cent of the years, respectively, in the two areas. The errors would have been entirely in predicting late blight years when the disease failed to

develop in epidemic proportions. Cook (54) also described a method for rapid determination of the beginning of the critical blight period by means of a chart of the moving 7-day average total rainfall and moving 7-day average mean temperature for the blight and nonblight years. From 1947 to 1950 accurate predictions of the blight situation in eastern Virginia were made by Cook (52, 54) and Nugent (142, 143). It was estimated that a saving of $2,000,000 was gained by eliminating unnecessary spraying and dusting in eastern Virginia in 1947 alone.

In the northeastern region, Hyre & Horsfall (94) collected and analyzed G. P. Clinton's records on the occurrence of late blight from 1902 to 1937; also subsequent records of the Connecticut Agricultural Experiment Station, and rainfall and temperature data for Hartford and New Haven, Connecticut from the Climatological Data of the United States Weather Bureau. Using Cook's method (50, 51, 54), weekly mean cumulative rainfall in inches and the critical rainfall line were determined for the 49-year period prior to 1950. They concluded that it is possible to predict the probable occurrence of late blight on potato foliage in Connecticut on an area basis with about 80 per cent accuracy by means of the critical cumulative rainfall line and weekly mean temperatures. These data corroborate Cook's findings (*loc. cit.*).

In 1954 Hyre (88) worked out a modification of Cook's method for forecasting late blight. It is an extension of the "moving" rainfall and temperature analyses which Cook had used. In Hyre's modification, each point is the total rainfall for a 7-day period, ending that day, viz., total rainfall for, say, June 1 to 7 inclusive is plotted on June 7; that of June 2 to June 8, on June 8, etc. Mean daily temperatures are combined and are similarly plotted as 7-day averages of the means so that each point on the temperature line would represent the average of the mean temperatures for the 7-day period ending that day. On this basis an outbreak of blight would be forecast by seven consecutive days favorable for blight. Favorable days result when the 7-day mean temperature is 77°F. or less, and the 7-day total rainfall equals or exceeds about 1.2 inches. Greater accuracy, however, was obtained when a similarly derived 10-day moving rainfall line was used. This prerequisite for so many consecutive favorable days for blight to threaten applies to the initial appearance of the disease. Once blight is present fewer favorable days are required for the fungus to sporulate and for new infections to result.

In 1955, Hyre (90) compared three methods for possible use in making actual forecasts of late blight of potato and tomato in northeastern United States. Two of the methods were based on relative humidity and temperature data. The other method uses the moving graphs of rainfall and temperature data that he had previously (*loc. cit.*) worked out. It was the most successful of the three methods and was very reliable for forecasting blight.

Hyre & Bonde (92) discuss the possibility of forecasting late blight of potato in northern Maine. Study of the records indicates that successful forecasting could be achieved by basing predictions on an analysis of rainfall and temperature by means of the "moving" graph system already discussed. However, 10-day, instead of 7-day, moving precipitation records and 7-day

moving temperature figures would give a higher degree of accuracy in predictions. In 1955, actual forecasts of the incidence of late blight in Aroostook County, Maine, were made for the first time with very good results. Similar success has also been achieved in the 1956 season and these forecasts will be continued. It is hoped that routine prediction of late blight occurrence in this important potato-growing area will lead to increased efficiency and stability in the production of the crop and in the application of control measures when needed.

The conditions and schemes, so successful in the East for predicting blight incidence and development, seem not to be applicable to the Midwest, including the north-central region. Hyre and Horsfall attribute the discrepancy to difference in occurrence of primary inoculum potential from year to year, which is fairly uniform in the Atlantic Coast area but varies a great deal in the reasonably blight-free areas of the Midwest.

However, Wallin (206) and Wallin & Waggoner (211) have worked out bases suitable to midwestern conditions and have made successful experimental forecasts that achieved an extremely high degree of accuracy in predicting both the initial appearance and secondary spread of the disease. Their methods are a modification of Beaumont's English rules to suit midwestern American weather conditions. They are based upon the relationship of the reproductive process of the late blight fungus to the duration of certain periods of temperature and rainfall, or temperature and relative humidity. They also made some attempts to predict blight intensity on the basis of duration and frequency of favorable periods for its development. They showed that successful initiation of late blight can occur when temperatures are 75°F. or less each day for 8 days and the relative humidity is 90 per cent or more for 10 hr. each day (211). Wallin (206) demonstrated, with experimental forecasts, that conditions can be considered favorable for sporulation, spore germination, and host infection if the temperature and relative humidity for 10 or more consecutive hours is 70° F. or less and 95 per cent or more, respectively.

A major point of difference, therefore, between eastern and midwestern criteria is that blight conditions can occur without measurable rainfall in the Midwest. In the north-central region it was found that rainfall and temperature are not sufficient to indicate favorable conditions for blight development (209, 210, 212). Here atmospheric humidity seems to be the important factor, and it has been shown that the hygrograph provides a more complete account of humidity conditions in relation to late blight occurrence than do rainfall data alone, because this machine records the humidities associated not only with rainfall, but with dew deposition, fog, and saturated atmosphere as well (212).

Fundamental research.—Important fundamental studies have been undertaken to support the forecasts and, through them, to promote effective utilization of control measures. Spatial distribution of the fungus, temperature relations of the host plant, and pathogenicity of various isolates or existence of strains or both have been the main subjects.

Waggoner's experiments (204) showed that distribution of sporangia of *P. infestans* vertically, crosswind, and downwind in the atmosphere is caused by the turbulent transfer of sporangia from a point source; that sporangia evidently move with the air movement; and that distribution occurs from plant to plant. In 1950 Wallin & Waggoner (211) had also established that spread of infection was gradual, from plant to plant, rather than by discontinuous jumps from the center to the periphery. The greatest distance of appearance of blight from the source of sporangia is on the downwind side and towards obstructions to air movement. The percentage of leaflets with blight lesions showed a rapid initial decrease and became progressively smaller as the distance from the source of inoculum increased. Thus, spores from close-by sources, such as cull piles, are more important in the spread of late blight than spores carried from a distance.

As to the measurement of dispersal factors, in 1945 Gregory (73) derived a mathematical model for the deposition of air-borne spores, and Waggoner (204) proposed a mathematical formula for estimating the percentage of leaflets diseased, in which the percentage of leaflets diseased is a function of parameters that depend upon characteristics of the sporangial dispersal process. Waggoner's model differs from Gregory's in taking into account the damping of vertical turbulence by the earth's surface, and makes explicit the relation between spore deposition and infection.

Temperature relations of the fungus have been studied. Viable sporangia of the late blight fungus were found to be adrift in the air at least until noon, which extends the known period during each day in which infection can take place (211). Viable sporangia were found on potato and tomato foliage during the afternoons when temperatures were between 80° and 90°F., and during the following evening as well, proving that the late blight fungus can withstand lower humidities and higher temperatures than was previously supposed. Wallin (207) also found that viable sporangia are produced within 6 hr. on potato foliage during the hottest part of the day, a period usually considered adverse to sporulation.

Techniques.—In some of the studies conducted under this program techniques and equipment to be used constituted an important part of the problem. For example, a special sampling method was developed to obtain a correct estimate of the number of viable sporangia of *P. infestans* in the air at various times of the day (211). As already mentioned, a mathematical hypothesis was worked out for measuring the spatial distribution of sporangia in various directions from a point source (204). Investigations on strains by Wallin & Waggoner (205) required a precise separation of the effects attributable to each single factor among the various factors influencing disease. From a thorough review of previous work on the subject of races in fungi, they defined four types of races according to the particular one of the four factors: host, pathogen, environment, and time, correlated with significantly different amounts of the disease. The definitions and mathematical expression will be useful in all similar studies.

As a record of dew deposition was often required in these late blight microclimatic investigations, and since no instrument for the automatic recording of dew existed, an instrument was invented. It is called the Wallin-Polhemus Dew Recorder. This new instrument records the onset and duration of dew deposition and is simple in construction and operation. Its use will add greatly to knowledge concerning the relation of dew to secondary infection (208).

For similar research, principally in Great Britain and on the Continent, the reader is referred to the section, "Research Contributing to Forecasting," at the end of this paper.

Bacterial Wilt of Sweet Corn

Study of the effect of environmental conditions on plant disease development provided the basis for reliable prediction of outbreaks of bacterial wilt (*Bacterium stewartii* E. F. Sm.) of sweet corn. Stevens (173) in the United States noted the association of a warm winter, or a series of warm winters, with serious outbreaks each following spring and summer. A warm winter furnishes ideal conditions for survival of the adult flea beetles in which the pathogen overwinters.

During the latter half of the 1930's, annual experimental general forecasts (174) were published based on "winter temperature indexes," i.e., the sum of the monthly mean temperatures for December through February. An index below 90 usually indicated that the disease would be absent; over 100 would indicate presence of the disease in destructive amount; and between 90 and 100 would indicate intermediate conditions. These general forecasts were discontinued after the accuracy of the predictions was established, since the method required no complicated organization of data or calculations and could very easily and more efficiently be applied directly to specific locations by State pathologists.

Incidence of the disease in Illinois in 1949 and 1950 (20, 152, 155) confirmed the correlation between warm temperatures during the preceding winter and abundance of infection. Boewe's forecast (20) that, because of the warm winter of 1949–50, the disease would probably occur much farther north in the State in 1950 and be much more destructive than it was in 1949. proved accurate. Moreover, observations indicated that the leaf blight phase of the disease in field corn could also be predicted from winter temperatures. In 1952, 1953, and 1954 the development of both early-season wilt, especially in sweet corn, and of late-season leaf blight coincided very closely with the fourth, fifth, and sixth "Stewart's disease prospects" prepared by Boewe (21, 22, 23). In 1955, occurrence of both phases was generally as predicted, again especially on sweet corn (24). Boewe comments that other unknown factors, in addition to winter temperatures, may affect the survival of the vectors and consequently result in some deviation of actual from predicted occurrence. Boewe's 1956 forecast (25) was also correct.

Highly accurate forecasts have been made in recent years in New Jersey

and in New York, reports of which have appeared in the Warning Letters issued by the Plant Disease Epidemics unit. These have been useful and accurate.

In New York (records for Tifton, N. Y.) it was found during the past eight years that bacterial wilt was severe when winter temperature indexes were 90° or over, and moderate or light when the indexes fell below 90°F.

Blue Mold of Tobacco

From a study concerned with the incidence and build-up of tobacco blue mold (*P. tabacina* Adam) in the tobacco-growing regions of the United States, two strong indications as to the amount of damage and the effect of weather in relation to the yearly fluctuation in disease severity emerged. During a survey of 2000 plant beds scattered throughout the tobacco-growing area in the South (122), it was noted that the amount of damage caused by blue mold in a given locality seemed to be related to the earliness of the first signs of infection. In addition, there appeared to be a close relationship between above-normal January temperatures in the southernmost tobacco-growing States and the build-up of the disease. Analysis of weather records of January temperatures at five southern locations in this region showed that yearly fluctuations in intensity of the disease (122, 126) have closely followed the deviation from the normal of January temperatures for the years 1931 through 1951. Earliness of appearance in the Quincy, Florida, and Tifton, Georgia, areas occurred in those years when above-normal January temperatures approached the optimum, 62°F., for mildew infection. Continuance of favorable weather conditions, of course, aided the development of the disease. In those years when January temperatures were lower, blue mold appeared late and caused little damage, and even if favorable weather conditions prevailed later in the year, actual damage was slight.

In 1951, Miller (in mimeographed Warning Letters) predicted that if a positive correlation does exist between the mean temperatures in January and the amount and severity of blue mold developing later, then the 1951 season should be about average for the disease. Since the factors involved in this apparent relationship of January temperature and blue mold development are not known, the statement was based on strictly empirical information. However, there had been no major exceptions to this rule during the prior twenty years for the area under consideration. In the following years, 1952 to 1956, January temperatures were again examined but no predictions were published. The range of above-normal January temperatures in 1952 and 1953, the very small amount of deviation from the normal in 1954, and the below-normal temperatures in 1955 and 1956 could be easily compared for possible correlation by anyone interested in determining the relationship.

Stover & Koch (175) point out in their study on the incidence of blue mold in Ontario, Canada, that severe outbreaks of blue mold in that Province are dependent upon spore showers originating in the Kentucky-Ohio tobacco-growing regions of the United States. In the epiphytotic years of 1945 and 1946, it was noticed that heavy spore showers in Ontario were closely asso-

ciated with severe outbreaks in the Kentucky-Ohio area. These unexpected and widespread spore showers usually cause general outbreaks before adequate control measures can be carried out. Complete routine spraying and dusting might control the disease effectively but the expense would be exorbitant. Since the interval between the appearance of blue mold in the Kentucky-Ohio area and its appearance in Ontario is approximately two to three weeks, complete information on the severity and build-up of the disease in Kentucky would make possible the establishment of a warning system based on the probability of spore showers and on the current local weather conditions most likely to favor development of the fungus in Ontario.

Analyses of this type have practical applications as a basis for prediction prior to more elaborate and precise research in epidemiology.

Downy Mildew of Lima Bean

Successful forecasting of the occurrence of downy mildew (*Phytophthora phaseoli* Thaxt.) of lima bean has been initiated and achieved during the past four years (1952 to 1956) in Delaware (89, 91, 93). The technique used in making these forecasts was the same as that for forecasting late blight attacks, viz., by the "moving" graph method. Two like measurement methods seem successful, i.e., 8 to 10 consecutive favorable days with an average temperature of not more than 77°F. and 10-day total rainfall equalling or exceeding the 10-day average. The second method requires 9 to 10 consecutive favorable days, when the maximum 7- (possibly 5-) day temperature does not exceed 68° to 70°F. and the 10-day total rainfall equals or exceeds the 10-day average.

Since these forecasts have been highly accurate in 1956, it is proposed to extend the area of forecasting to the eastern shore of Maryland. These forecasts render a real service to growers as fungicides are not usually applied to lima beans except for the mildew, and growers are reluctant to apply unnecessary sprays because of the relatively low margin of profit on the crop.

Wheat Leaf Rust in the United States

About fifteen years ago K. Starr Chester developed a system for forecasting the severity of wheat leaf rust [*Puccinia rubigo-vera* (DC.) Wint. f. *tritici* (Eriks. & E. Henn.) Carl.][2] in Oklahoma, which has been distinctly successful (39 to 42, 44 to 48, 217 to 222). The basis for the forecasts was a thorough study of the overwintering and early spring renewal of the rust as related to late winter and early spring weather conditions.

The reasoning behind the forecasts is that the principal source of rust in Oklahoma is inoculum from overwintering local infections. Secondly, the weather of December through February, and especially of March, is critical in determining the spring renewal of the rust. Thirdly, the level of rust intensity on April 1 is the principal factor in determining destructiveness from April to harvest in June, since the number of generations of rust in-

[2] *Puccinia recondita* Rob. ex Desm. considered oldest valid binomial by Cummins & Caldwell (cf. *Phytopathology*, **46**, 81–82, 1956).

crease is limited in this period of time and, finally, the weather in Oklahoma is rarely a factor in limiting rust development after April 1.

The amount of inoculum present on April 1 is determined in two ways. Overwintering and early spring development is closely observed at Stillwater, Oklahoma, by periodically counting the number of rust pustules on 1000 tillers of a susceptible variety (Cheyenne) of wheat. Two to three thousand pustules on 1000 tillers of Cheyenne on April 1 indicates that there will be approximately 5 per cent crop loss by harvest time, which has been found to be about average or normal for Oklahoma. These pustule counts are then supplemented by a statewide survey during the last few days in March to determine if conditions at Stillwater have been representative of the state as a whole. If not, the forecast is adjusted in accordance with the data accumulated on the survey.

To date, fifteen annual forecasts have been made and every forecast on these bases has been accurate for Oklahoma, and each forecast has also usually been applicable throughout much or all of the wheat areas to the north, except for one year. In 1952, the forecast was for a 7 to 10 per cent loss in the crop, but rust development was halted by a severe hot, dry period occurring in the second and third week in May. Save for this one dry year, the principal variable factor in leaf rust epidemiology in Oklahoma has been found to be the amount of inoculum present over the state at the beginning of the period (April 1). These forecasts have been invaluable in helping farmers and grain elevator operators to plan for harvest and disposal of the crop or its abandonment in favor of spring-planted summer crops.

Apple and Pear Scab

Another program of research and forecasting was related to predicting the time of development and severity of apple scab (*Venturia inaequalis* (Cke.) Aderh. = *V. i.* (Cke.) Wint. apud Thuem.) and pear scab (*Venturia pirina* Aderh.) for the efficient employment of spray materials and the prevention of crop loss.

Environmental factors favoring development of the fungi were considered important in promoting a spray program. Rainfall was the first weather factor studied. Bremer (33, 34, 35), in his analysis of meteorological data, found a distinct connection between rainfall during the first ten days of May and the incidence of scab in the Proskau district of Silesia. The amount of rainfall in this period before the opening of the blossoms seemed to determine the incidence of the disease, heavy rains at this time generally denoting a severe epidemic the following summer.

About twenty-four years later Louw's studies (109) indicated that increased incidence of apple scab in South Africa in the middle 1940's was not correlated with the corresponding higher rainfall and that other factors might predominate over the effect of wet conditions. In the winter-rainfall area of the Cape region of South Africa, initial source of infection is from fallen infected leaves (110). Louw pointed out (111) that winter temperatures ex-

erted a dominating influence on scab development in South Africa, apparently accounting for variations in severity from year to year, with severe outbreaks coinciding with relatively low winter temperatures. These data provided a basis for estimating in advance the probable severity of the disease in any particular season and made it possible to warn growers in localities where severe outbreaks would be likely to occur.

Information obtained in Germany from the study of the effect of natural environment on disease incidence and development led to proposals for prediction of appropriate dates for spraying according to ascospore discharge and bud development (83, 84, 86, 96). Spores were trapped under both laboratory and out-of-doors conditions, and the results compared from time to time. In Holz' experiments (83) the first spores were trapped in the laboratory eight days prior to the first spores trapped in the open following rain. An immediate notification of the laboratory results to the growers would thus enable them to utilize the interval regularly elapsing between indoor and outdoor ascospore production for the beginning of the first and important fungicidal treatment.

From seven years' observations, Holz (84) found the average dates on which perithecia ripened in Altenland, Germany. Temperature aggregates, based on mean daily temperatures, were calculated for each year from the first or fifteenth of each month between November and April to the date of ripening of perithecia. Temperature before the first of March had no appreciable effect upon ripening of the perithecia. From March 1 onwards until perithecia ripened, the mean day-temperature aggregates were almost constant during the seven years. They were, on the average, 105°C. whereas temperature aggregates from earlier or later dates differed considerably. Holz concluded that the sooner a mean day-temperature aggregate of 105°C. is reached after the first of March, the sooner the perithecia will ripen. Jahn (96) tested the practicability of Holz' methods for prognostication of the beginning date of ascospore discharge on which to base the spraying schedule. Since his observations confirmed those of Holz', that under outdoor conditions spore discharge commenced a few days after the initial discharge of spores on overwintered leaves tested in the laboratory, Jahn concluded that appropriate dates for spraying could be predicted reliably on this basis.

Hus (86) objected to using ascospore discharge as a signal for spray warnings because of six points: (*a*) ascospore discharge in one locality does not necessarily coincide with the same process in other places where rain may fall on different dates during the critical periods in April and May; (*b*) unfavorable spray conditions might exist at the time of ascospore discharge, and once infection is established, it is very difficult to suppress; (*c*) treatment of large areas required days so that even in fine weather part of the orchard may be reached too late; (*d*) spraying could not be carried out on a cooperative basis; (*e*) if spraying is done as soon as perithecia mature, too long an interval might elapse between treatment and ascospore discharge under rainfall, the exact date of which can seldom be predicted several days be-

forehand; (*f*) in mixed plantings perithecia might ripen sooner on the leaves of one variety than on another, and, therefore, several ascospore discharges would have to be considered in fixing the spraying dates.

In France, Darpoux (60) points out the use of ascospore discharge values in connection with fungicide application tests at different growth stages. Warning threshhold values for mature perithecia after which there is serious danger of primary infection were determined. In his experiments in 1952 and 1953 at Versailles, using a modified version of Vuittenez' apparatus (cf. *Revue de Pathologie végétale et d'entomologie agricole de France*, 28, 118–25, 1949) for reception of spores emitted by mature perithecia, he found that the warning threshhold was reached at an ascospore emission of about 1000 to 1500 per hour when the pear tree was between full bloom and fruit swell.

Panjan (149) and Mirić (132) conducted experiments in Yugoslavia for the purpose of establishing a forecasting service for control of apple scab. Mirić's results showed that well-timed applications of control formulations can prevent early and late infections on the trees.

In Italy, also, experiments were conducted (151) with a view to determining the practicality of establishing a system of warning stations for apple scab control. By using exposed-slide spore traps in orchards and by watching the progress of perithecial maturity in the laboratory, it was found possible to follow the processes of disease development and forecast the period of ascospore discharge in time to permit growers to apply sprays.

Warning services for scab control.—Several warning services are now being conducted abroad for apple scab control. In a report of the Cawthron Institute (37), New Zealand, mention is made of a notification service for the benefit of growers in the Nelson area in connection with scab control. In Australia a warning service is conducted for the prevention and control of apple scab (114). In Norway investigations were to be conducted on ascospore dissemination during 1951 to 1953 with the purpose of establishing a warning service for apple scab control (98). We have found no mention of the actual organization of this service in the literature. Fruit spraying in England is based on a calendar which depends on the stage of development of the leaf and flower buds, and no apple scab warnings are considered necessary (75).

In Holland, an apple and pear scab spray warning service (158, 181, 182), timed according to the dual criteria of ascospore liberation and bud development, was initiated in 1948. This service is based on laboratory examination of leaves collected shortly before the first ascospore discharge and examined after 4- and 24-hour intervals. Six to eight days after the beginning of profuse ascospore discharge under the warm and humid conditions prevailing in the laboratory, abundant spore "flight" may be expected in the open. Eight years' observations in five provinces revealed no material discrepancies in the dates of ascospore liberation. Also, the stage of development of the buds was taken into consideration. Since bud development is dependent upon weather conditions, close daily contact is maintained between the Meteorological

Institute and the Plant Protection Service. The spray warnings are promptly included in the wireless weather reports and distributed in other ways as well. Continued research (198) is being carried out under conditions favoring infection.

Vullings (202) reports a severe apple scab epidemic in 1949 in Holland that resulted in a loss of 1,000,000 gulden to growers in the Limburg region. According to the author, crop failure is nearly always attributable to unduly long intervals between treatments. Omission of one treatment at the decisive blooming period cannot be retrieved by three or four summer sprays.

While European countries were exerting vigilance in preventing losses from scab by various methods of prediction, American and Canadian pathologists were similarly concerned. Spray information services, with special reference to the control of apple scab, were organized in New York, Delaware, Ohio, Indiana, Illinois, Kentucky, Wisconsin, West Virginia, western and eastern Canada (1, 3, 56, 57, 63, 64, 68, 80, 85, 100, 107, 144, 150, 153, 162). For the most part, these programs were based on a careful study of the relation between weather and disease. By studying the development of the fungus under both laboratory and field conditions (216), it was possible for these workers to determine in advance when and under what conditions apple scab is likely to occur in their particular regions. This knowledge, plus information on the stage of fruit bud development and conditions of tree growth, is correlated with the local weather forecasts in the United States and Canada. On these three factors, in combination, spray warnings are based and issued to growers.

The organization of all these spray warning services follows somewhat the same pattern. From observations made by pathologists, entomologists, and county extension workers, growers are notified by letters or cards as to the most opportune time for spraying, oncoming rain periods, recommendations concerning materials and strength of spray materials to be used, and modifications in the sprays for special varieties of apple. The alerted grower waits for messages by telephone or other rapid means of communication as to the exact time for spraying or dusting. The radio has been an important aid in disseminating this information, and messages so sent are keenly awaited and followed by growers, These rapid messages are usually confirmed by card sent through the mail the next day.

The grower, in many cases, keeps records of the success or failure of his spraying. Percentage of clean fruit and yields are also studied. When loss is caused by inadequate spraying, recommendations for future seasons are made by trained specialists for correct application of sprays in his particular orchard.

In eastern Canada the Province of Ontario began its spray service in 1924 (85). The service covers two districts, one for the Niagara district which included tender fruits such as peaches; the other for the rest of the province is chiefly concerned with apples. Spray recommendations are made under the direction of staff members of the Ontario Agricultural College, and the details

of the operation are carried out by local supervisors, usually one to each county. The service is free to any grower, the one requirement being that the the grower has adequate spray machinery.

In the Province of Quebec an orchard spray service was inaugurated in the spring of 1929 by the Provincial Department of Agriculture (69). Spray information is based on microscopic examination of perithecia on old leaves collected at two- and three-day intervals. The first spray depends on the time of maturing of perithecia and ascospores. Subsequent sprays are recommended according to host development and weather conditions. Godbout (68) reports that, since it is now known that ascospore discharges occur each time there is sufficient rain, cards are sent to growers in advance of the time when each spray should be applied. These cards contain information on spray formulae to use and various remarks regarding treatment.

In western Canada a spray warning service for apple scab was instituted on a small scale in the Kootenays in 1929 (162). Growers are advised to apply the first spray just as the ascospores on old leaves reach maturity. If the first application is postponed until pink stage (when flower buds show pink), secondary infection occurs. In the second report on this service, which uses time of spore maturity and discharge and weather forecasts as criteria, it was noted that meteorological observations were of considerable value in fixing the time of the first spray and in determining dates of probable heavy infection, but that reduction in the number of sprays seemed to be an undue risk (63).

Downy Mildew of Grapes

Italy.—Almost concurrently with the organization in Europe of a forecasting system for apple scab control, a vine spray warning system was organized in western Europe for the control of *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni. As in the case of apple scab, the forecasting of vine mildew was set up on the basis of the relation of weather conditions to the development of the fungus.

Fortnightly bulletins were compiled from meteorological data on conditions favoring epidemics of vine fungus diseases in several provinces of Italy and in Sicily (36, 117, 133, 200, 201). Interest in broadening the scope of these antimildew observatories has continued through the years (6, 8, 116, 161). Reports for each area are sent to a central pathological station, where preliminary instructions for preventive treatment are issued as soon as the information received indicates favorable conditions for germination of the winter spores and for the first infection of the vines. Later, appropriate treatment is recommended at each successive stage of attack in accordance with rainfall and other weather factors (200, 201).

Dew point estimation, as a basis for forecasting, interested Italian workers. In the vicinity of Perugia (55) records were taken on dew point, or that temperature at which atmospheric moisture is condensed, thus bringing about favorable conditions for the germination of zoospores. If the dew point was higher than 12°C., the lowest temperature at which germination

takes place, an outbreak of vine mildew might be expected. From his experiments Corneli concluded that the presence of condensed moisture was the predominating factor conducive to an attack of mildew, particularly in central Italy where average daily temperature in late spring and summer favors infection, and that the safest procedure for effective forecasting would be to couple direct observation of conditions leading to the condensation of moisture with thermometric and psychrometric data on atmospheric changes.

In using Müller's incubation calendar (*loc. cit.*, vine mildew forecasting in Germany) for the determination of treatments, Baldacci (55) found that in northern Italy the pathogen required a shorter incubation period during May than was allowed by Müller's calendar. High atmospheric humidity was determined to be an important factor in shortening the incubation period. Incubation periods prevailed in every locality along the northern slope of the Appenine chain and, therefore, the incubation calendar could be used to advantage in northern Italy and Tuscany (7). However, in regions where fog and dew are prevalent, applicability of the calendar is limited to the first treatments.

The effectiveness of forecasting systems for vine mildew is reflected in the satisfactory results obtained with proper application of control measures (67, 118, 119, 154, 163, 164, 178).

France.—The operation of the French vine mildew warning service paralleled the Italian service, with five stations in operation, viz., Bordeaux, Montpellier, Clermond-Ferrand, Avignon, and Antibes. The French forecasting service was another based on the interrelation between meteorological factors and disease appearance and development. Dew, mists, and nocturnal rain were found to be factors favoring infection (95).

The progress of oospore germination was determined under local conditions from specimens collected at observation posts during March, April, and May (32, 166, 167). Prediction of the primary period of infection is based on presence of active oospores, temperature above 11°C., and a rainy period keeping the surface of the soil wet for several days. Germination of overwintered oospores under natural conditions is the signal for the spring renewal of the disease. By following the temperature and rainfall, and by knowing the abundance and germinative capacity of the spores, it was found possible to predict the extent of first infection. Secondary infections were found to be dependent upon active conidia, rain, and a temperature over 8°C. Persistence of water on the leaves for six or more hours at a temperature between 11° and 20°C. will inevitably induce infection. Number of treatments required for control is determined by extent of primary infection, coincidence of conidial formation with rain, amount of rainfall in June and July, and the critical stages of vine growth. In the calculation of time for incubation, the appearance of infection spots can be determined with greater accuracy if only 6 hr. in which the relative humidity is over 60 per cent are considered. Also, Branas (31) has pointed out that temperature does not appear to be the important factor locally in determining length of incubation period, but that the water content of tissues is also important. It is

suggested that some relation probably exists between severity of attack, size of lesions, duration of incubation, and the degree of turgescence of the tissues (166).

At the Montpellier Station another type of warning (17) is based on adapting frequency of spray applications to growth rate of the vines. Its value lies in not permitting branch tips to remain too long without treatment. Intervals between spray applications, shown as length in centimeters, depend on host vigor, number of attacks already experienced, and the local rainfall.

In its early days the warning service was conducted by relaying warnings or reports by visual or sound signals (38). Later the reports were sent as code telegrams at minimum cost to subscribers, giving information on spray warnings, the weather, appearance of pests, etc. Excellent practical results have been obtained under the French spray warning service, both in the reduction of number of spray applications and in the application of control measures only when needed (59).

An interesting note on fungus ecology in relation to forecasting is the appearance of *Gnomonia veneta* (Sacc. & Speg.) Kleb. on *Platanus occidentalis* L. preceding the first attack of vine mildew by several days. This regular appearance can be used as an index of approaching vine disease (58).

Germany.—The vine spray warning system in Germany was based on the "incubation calendar" created by Müller in 1913 (135, 137 to 140). This calendar acts as a guide to timely spraying, according to temperature and moisture conditions and their effect on the future development of the disease. The system has achieved a high degree of reliability and by the use of the calendars spraying times were very accurately predicted for the period 1913 to 1938 (135, 137, to 140).

Greece.—The question of agricultural warnings in Greece has undergone investigation (4, 224). At the L'Institut Phytopathologique Benaki it was planned for a long time to study certain phases of the biology of *Plasmopara* in the different vine-growing regions of the country, with regard to the possibility of organizing warning stations and applying, in Greece, the methods used and approved in other grape-growing countries, particularly France. This plan was first put into effect in 1949 and the first observations were made, in Peloponnesia, in the region of Corinth, in 1950. This one year's work of correlating weather conditions and the development of *Plasmopara* with the stages of vine growth, particularly in the critical stages, showed that three applications of dusts were sufficient for protection of the vineyards in this region. This amounted to a reduction of more than 50 per cent in number of treatments, with consequent economy of copper sulfate and saving of the growers' time and energy. It was planned to continue the study with the hope of establishing, as soon as possible, the warning stations necessary to protect the vineyards with a degree of certainty. We have not been able to find any subsequent reports on this situation in the literature available to us here.

Rumania.—A note in the literature advocates a coordinated spray-warning system for vine-growing areas in Rumania (196).

Russia.—A phase of the vine mildew forecasting work in Russia was based on the "incubation calendar" of Müller, modified to suit different growing areas and climatic conditions. Oltarjevski (145, 146) constructed a preliminary "incubation curve" of the vine mildew disease for the Derbend region but found that Müller's "incubation curve" would not be applicable under all sets of climatic conditions and should be corrected to suit the local conditions prevalent in different regions.

Modified formulae, suitable to environmental conditions in the vine-growing areas of the U.S.S.R. were worked out (169) in order to calculate treatment dates. A calendar of incubation periods was compiled for the locality to be studied, using temperature data and many years' records and the proposed equations.

Oltarjevski (147) and Merjanian & Lipetzkaya (121), in field and laboratory experiments on the effect of light (Oltarjevski) and temperature (Merjanian & Lipetzkaya), have also contributed much in reducing biological forecasting studies on *Palsmopara* to a mathematical basis. Oltarjevski (147) demonstrated that daylight had an inhibiting effect upon conidial fructifications when leaves exhibited the oil patch stage of the disease. Production of conidia could also be delayed by gradually increasing the intensity of the light to which leaves were exposed. Minimum temperatures between 10° and 13°C., if occurring just before sunrise, were found to influence production of vine mildew outbreaks. Merjanian's & Lipetzkaya's (121) work on lengthening or shortening of the incubation period was mostly concerned with temperature effects, and a formula for expression of the interaction of temperature and length of the incubation period was devised. Range was found to be within the limits of 13° to 24°C., or the average temperatures of Müller's curve.

Spain.—Clemente (49) describes an instrument, Präzisions Polymeter, which is used to indicate the opportune moment for treatment with fungicides. This instrument consists of a thermometer and hygrometer, the thermometer surmounting the round clock-like hygrometer. If the difference between the thermometric temperature and the humidity measured on the hygrometer is less than ten degrees, it is not probable that mildew will develop, but if the difference is above ten degrees, there is danger of infection. These observations are made an hour before sunset. The instruments are usually installed in open fields but are protected from the sun and climatic changes.

Switzerland.—According to Kundert (101) the peculiar climatic conditions in Switzerland make reliable forecasts of the incidence of vine downy mildew impossible.

Yugoslavia.—Results of experiments in 1952 to determine the possibility of establishing a central forecasting service to issue spray warnings for the control of vine downy mildew were definitely favorable (112). The requirements for the initiation and development of the disease were determined as: (*a*) temperature above 12°C. (optimum 25°); (*b*) rain for at least two days with at least 10 mm. rainfall; (*c*) leaves at least $\frac{2}{3}$ cm. in size (113). Results on forecasting vine downy mildew in Serbia in 1953 confirmed the conclu-

sions that spray warning services can be based on meteorological data (114).

Australia.—A warning service is conducted on downy mildew of grapes (*ibid.*, late blight section).

RESEARCH CONTRIBUTING TO FORECASTING

The scientific bases for crop loss prediction are greatly strengthened by research in many different phases of plant science and allied fields, even though specific objectives of these studies are often not directly concerned with forecasting. Important topics in this classification include studies on epidemiology and effect of environment on plant disease development; investigation on the physics of spore dispersal and the mechanics of disease spread; mathematical and statistical analyses of infection phenomena; estimation of disease intensity and the formulation of estimation scales; and appraisal or estimation of crop loss. The solution of many of these problems has, when applied to plant disease forecasting, increased our knowledge of cause and effect, explained the nature of recurring patterns of disease development, and helped pathologists in developing and applying adequate control measures.

The quantity and persistence of water deposits on plant shoots are important epidemiological factors. Hirst (82) in determining the amount and duration of such water deposits, designed an apparatus to record the amount of water deposited on plant shoots by rain, dew, and guttation, and to measure how long the surface remains wet. This apparatus, the design of which is given in Hirst (*loc. cit.*) was tested in the field in 1952 and 1953. The surface area of a shoot is measured and by calibration of the beam deflection caused by the water weight on leaf surfaces, the weight of water per unit area of leaf is estimated. Rain was found to be deposited rapidly, its persistence on the leaf depending upon weather conditions. Dew was deposited slowly over a longer period and dried up more rapidly. Guttation is thought not to be an important cause of surface water on potato leaves as it is on other plants. For estimates of the mean weight of water over other plant areas, such as petioles and stems, it would be required to use several balances bearing variously divided parts of the shoots. The use of this instrument in studying the relation of moisture conditions to potato blight can be readily visualized, as here, no doubt, the number and viability of *P. infestans* sporangia may depend to a large extent on the length of the period during which viable sporangia and water droplets co-exist. [See also Wallin's work (207) on viability of *P. infestans* sporangia under the section on Potato and Tomato Late Blight in the United States.]

Understanding of disease spread can be gained and reliable bases for predictions can be built up from a knowledge of how spores are dispersed (19, 26, 76 to 78, 87, 171, 172, 213, 215), especially from studies on the mathematics and methods of estimating dispersal (74, 183 to 186, 203).

In Germany, Schrödter (171), working on the problem of spore dissemination in relation to mass exchange and wind, found that both height and duration of spore flight are independent of wind. Distance is the only factor influenced by wind. By means of equations for various exchange coefficients,

the height and duration of flight of different-sized spores was calculated, together with distances covered at wind velocities from 2 to 10 m. per sec. Minute spores were found to be capable of traversing considerable distances when winds of moderate turbulence and low velocity prevailed. Large spores could be carried for several hundred kilometers under influence of high wind speeds and considerable turbulence, e.g., the author notes dissemination of *P. infestans* spores for distances exceeding 100 km. in a few hours under rainy and windy conditions conducive to producing an epiphytotic.

Spore concentration in the air and the deposition of spores has been investigated by several workers with a view towards correlating the number of spores and patterns of deposition to appearance of crop diseases. Hyre (87) used the standard pollen gravity sampling device with vaseline-coated slides placed in horizontal, vertical, and 45° angle positions to determine (*a*) the number of spores in the air under different environmental conditions, (*b*) the distance these spores may be disseminated and still remain viable, and (*c*) whether control measures could be delayed until the presence of the inoculum in an area is established by means of these spore traps. Effectiveness of the slides for trapping sporangia of fungi (particularly those causing downy mildew of cucumber and lima bean) was negated by various factors, including principally the inefficiency of the traps themselves for accurate measurement of the number of spores per specific volume of air, and, secondly, the confused picture of spore deposition due to deflecting obstacles on the traps, such as supports, clamps, etc., which might be in the path of the wind-directed spores. Hyre therefore concluded that the spore traps had little practical use as aids in forecasting the diseases included under the Crop Plant Disease Forecasting Program of the U. S. Department of Agriculture.

According to Gregory (76, 78) spore concentration was only one factor in determining deposition of spores, noting that particle size, wind speed, and the size and orientation of the trap surface are all very important. He found that records from surface traps were too difficult to interpret in terms of spore concentration per unit volume. The Cascade Impactor (120) proved to be an efficient instrument for volumetric sampling of the outdoor air spora for visual inspection under the microscope. Spore deposits are too dense, however, unless the slides are changed frequently, consequently the apparatus possesses only limited usefulness for continuous records but it is valuable for spot sampling.

With the Hirst automatic volumetric spore trap (81) the diurnal changes in air spora can be determined with an error of about ± 1 hr. This automatic volumetric suction trap contains a clockwork mechanism that moves a vaselined slide past a narrow (14×2 mm.) orifice at 2 mm. per hr., with the spores entering the trap being deposited on a 24-hr. trace 48 mm. long. A wind vane on the mounting directs the orifice into the wind. This precise instrument provides a high degree of accuracy for the study of kinds of spores and measurement of rates of deposition, because "impaction" efficiency is increased by artificial acceleration of a narrow air stream bearing representative spore loads towards a sticky surface, and changes in atmospheric spore content can be closely correlated with weather changes and to

variations in wind speeds at different times of the day by the known, measured movement of the slide across the orifice. Trap efficiency was calibrated by Hirst at each of four external wind speeds and two rates of suction through the orifice in a wind tunnel under conditions of controlled turbulence.

Waggoner (203), working on the aerial dispersion of *P. infestans* spores, derived an expression for spore deposition as a function of distance, x, from a point source; of source strength, Qo; and of the proportion, p, of the spores settling out as spore clouds passing over a unit area. His hypothesis for spore concentration was transformed into a formula for disease distribution:

$$D = \frac{0.135p(Qo/k)}{x^{15/8}} e - 6.78px^{1/8}$$

This formula was tested by field experimentation on the proportion and pattern of disease development on potato leaflets around a primary source of inoculum. The observed pattern of disease occurrence, when compared with Waggoner's theoretical parametric values, indicated the validity of his hypothesis.

Gregory (74) has drawn attention to the possible misinterpretation of results that may come about when per cent present or absent of an organism is used as the measure of disease incidence. To infer gradients of spore density or numbers of insect punctures, it is necessary to apply a multiple infection transformation (73, 176), as tabulated for ecological studies on *Ribes* by Fracker & Brischle (66). This is essential when more than 20 per cent of the plants are infected (79). When the data measure disease factors, for example underdispersion or overdispersion which are not randomly distributed, it is also necessary to find the correction factor, such as Blackman's K. For detecting overdispersions, or aggregation, which gives K greater than unity, Van der Planck (183) has provided a useful test employing the following formula for determining the number of random groups of two adjacent plants:

$$d = \frac{1}{n} \mu(\mu - 1)$$

when n = number consecutive plants examined in a sequence over a uniform area, μ = number of plants diseased. Area gradients of disease are to be avoided by dividing the field into small blocks and applying the method to each separately.

Large (102 to 106) investigated the problem of interpretation of disease measurements and the relation of these measurement curves to the development of forecasting in Great Britain. He discusses (102) some of the uses for blight-progress curves obtained by the use of the estimation key prepared by the British Mycological Society (134). From "half decay," or 50 per cent date, and the slope of a time-percentage curve, the whole course and timing of an epidemic can be defined. By inspection at 10-day intervals from time of earliest date at which outbreaks are likely to occur in a locality, the starting point being taken as 0.1 per cent blight in the key (Moore, *loc. cit.*), the course of a blight epidemic can be obtained. Four or five observations over the course of time will suffice to establish the general sweep of the

blight-progress curve. Grouping these progress charts would provide data for the study of regional and seasonal differences with eventual zoning of the whole country for relative earliness or lateness of the course of blight. Blight-progress curves have been shown to be useful in estimating loss of yields in the crop, indicating when protective spraying would have effect upon yields and timing of culm destruction in relation to tuber lifting and the prevention of tuber infection.

Here we should like to recommend to the reader Chester's excellent analysis (43) of the importance, principles, problems, and techniques of plant disease appraisal, especially, perhaps, the chapter on "Techniques for determining plant disease intensity" and that on methods and accomplishment of disease forecasting, "Forecasting plant disease outbreaks and losses." The Supplement includes an extensive bibliography on plant disease appraisal and interpretation.

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SOME PROBLEMS IN PLANT VIRUS STUDIES[1,2]

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Introduction.—If we approach the subject of plant viruses from the biologist's point of view, rather than from that of the biochemist or biophysicist, several intriguing but unexplained problems come to mind. The natural method of spread of a number of viruses is one such unsolved problem. No doubt the discovery of unsuspected vectors such as mites has contributed something to its solution, but there is more in it than the existence of unsuspected arthropod vectors or unusual methods of spread, although it is true, for example, that it took many years to find the vectors of the phony peach and sandal spike viruses. The viruses of tomato bushy stunt and tomato black ring offer an interesting problem in transmission. The first was originally described in 1935 by Smith (1) and shortly after that seemed to disappear from commercial tomato crops, and was only once recorded in Britain during the intervening twenty odd years. Fairly extensive inquiry at the time failed to detect any insect vector. Now the virus has turned up again in Italy in tomatoes [Gigante (2)] and in garden petunia plants, also in Italy. A similar experience occurred with the virus of tomato black ring first described in East Anglia [Smith (3)]. No insect vector was discovered for the virus which undoubtedly spread in commercial glasshouses. This virus also seemed to disappear until it turned up again in the present year (1956) infecting, not tomatoes, but lettuce on quite an extensive scale. Now, since these viruses have been found infecting host plants out-of-doors, it is obvious that there must be a natural means of spread. The sudden appearance of a new virus disease in a single plant growing out-of-doors or even in the isolation of an insect-proof glasshouse has been observed on more than one occasion, and no explanation of this phenomenon has as yet been forthcoming. The virus of lovage mosaic was observed in a bush of this species growing in a private garden and, although the diseased plant was kept in close proximity to similar healthy plants, the virus never spread to these neighbouring plants. Indeed, although the host-range by mechanical transmission was wide, artificial infection of healthy lovage seedlings was extremely difficult [Smith & Markham (4)]. Another interesting case was the apparently spontaneous development of a necrotic half ring on the leaf of a tobacco plant in one of the insect-proof Cambridge glasshouses. Sub-inoculation to a wide range of plants showed that an undescribed virus, called "broken ringspot" by Smith & Markham (5), was concerned. The origin of this virus has never been traced.

[1] The survey of literature pertaining to this review was concluded in December, 1956.

[2] The following abbreviations have been used in this chapter: TMV (tobacco mosaic virus) and CMV (cucumber mosaic virus).

The universal occurrence of the paracrinkle virus in all potatoes of the variety King Edward is a classical example of the kind of problem under discussion. This virus is unable to spread to other potato varieties or to susceptible plants of different species, since it apparently lacks any method of natural transmission. A somewhat similar instance is the occurrence of the latent sugar beet virus which may be detected at certain periods of the year in a high percentage of sugar beet and mangolds. In this case, however, the virus presumably spreads with great facility but by what means is quite unknown.

Examples of Similar Viruses Differing Markedly in One or More Properties

There are many instances of this type of phenomenon; one of the earliest known is the occurrence of the two strains of aster yellows virus. The type virus occurs mostly in the eastern United States, while the other strain, sometimes called celery yellows virus, is found in California. The divergence of this virus from the type consists largely of slight differences in host range and in some additional insect vectors. The close relationship between these two viruses is demonstrated by the cross-immunity which exists not only in the plant, but in the insect vector also [Kunkel (6)].

Two very similar viruses, known as the New York and New Jersey strains, cause the disease of potato yellow dwarf. The difference between the two lies mainly in the fact that each has its own specific insect vector. There are also very slight differences in the symptoms produced on certain hosts like crimson clover and *Nicotiana rustica*. A parallel case is the existence of three beet curly-top viruses, the North American, Argentinian, and Brazilian types. Here again the disease caused in beets is similar but there are slight differences in the symptoms caused on some host plants, and the insect vectors are specific for each strain.

The viruses of tobacco necrosis [Smith & Bald (7)], of which there appear to be several, are biologically similar if not identical, but they are serologically different [Bawden & Pirie (8)]. Bawden (9) describes the behaviour of a virus which seems to be unique; this is the production of host-induced, reversible changes. The virus was found originally infecting cowpea (*Vigna sinensis*) in Nigeria and causing a systemic mosaic disease. In the tobacco plant this virus produced symptoms resembling tobacco mosaic, and was in fact serologically related to TMV. The same virus in Prince beans (*Phaseolus vulgaris*) was only distantly related serologically either to TMV or to the cowpea virus obtained from mottled tobacco leaves. The cowpea virus obtained from the two hosts had different electrophoretic mobilities and that from bean was inactivated much more rapidly by ultraviolet radiation. The original cowpea virus from tobacco produced on *Nicotiana glutinosa* lesions which resembled those produced by TMV but that from bean produced le-

sions which differed in size and appearance. Bawden could find no evidence that these host-induced changes in the virus were due to a mixture of strains in the original culture.

All these groups of similar viruses presumably existed for years before they were discovered and their relationships elucidated. Let us now examine some similar phenomena which have apparently developed during the time the viruses were under actual observation.

Some Recently Observed Phenomena and Their Bearing on the Problem

Gradual loss by a virus of insect-transmissibility.—The suggestion has been made on more than one occasion that at least some of the plant viruses, particularly those which multiply in their leaf-hopper vectors, were originally insect viruses which became by adaptation able to multiply in a plant host as well as in an insect host. If this is so, it is permissible to postulate that a virus can also lose an affinity for a host as well as gain one. This raises the question as to how permanent is the relationship between a plant virus and its insect vector. If changes in the environment or transmission to an unusual host can influence this relationship either gradually or suddenly, then we have an explanation for some of the problems in plant virus transmission. There seems little doubt that individual variation does exist among aphid vectors of the same species, and the suggestion has been made that races of the aphid *Myzus persicae* may exist which are unable to transmit the virus of spinach yellows, of which this species is the normal vector [Stubbs (10)]. However, this is an instance of variability in efficiency of the vector and does not explain a complete loss of insect-transmissibility on the part of the virus. On this there are two possibilities which come to mind, first, a long-continued separation of the virus from its insect vector so that the insect-affinity may be altered and secondly, a mutation in the virus which might be caused by abnormal environmental conditions or transmission to some unusual plant host.

As regards the first possibility, Black (11) describes how three isolates of the virus of potato yellow dwarf were maintained in plants without contact with insect vectors for 16½ and twelve years respectively. Four hundred and fifty of the leaf hopper vectors failed to transmit when fed on *Trifolium incarnatum* infected with these isolates. On the other hand, 433 control insects that had fed on *T. incarnatum* infected with fresh virus isolates from the field, produced 102 infections when tested in colonies.

It may be suggested that this virus, after its long isolation from contact with its insect vectors, mutated so that it lost its vector-relationship. Black (11) quotes also the case of the well-known virus variegation in *Abutilon thompsonii* which is not apparently transmissible by *Bemisia tabaci*, the vector of the virus in other *Abutilon* spp. Infected plants of *A. thompsonii* have long been propagated vegetatively because of the attractive variega-

tion of the foliage produced by the virus [Orlando & Silberschmidt (12)].

There is some evidence that a strain of cucumber mosaic virus, a derivative of Price's "yellow" strain (13), has also undergone a slight change in aphid vector relationships. After 20 years of continued mechanical transmission the virus is still aphid-transmissible but with difficulty as compared with newly acquired isolates of the type virus. This was first pointed out by Bhargava (14).

These are examples of a change in vector relationship presumably brought about by an artificial and long continued separation of insect vector and virus. No doubt other artificial conditions might also have a similar effect such as the growing of a virus-infected plant in an abnormal environment. This possibility is discussed in a later section dealing with a virus disease of *Arabis hirsuta*.

The potato viruses known as C and Y present an interesting example of variation in their vector relationships. Although these two viruses are so alike that there is little doubt of their strain relationships, virus C, unlike virus Y, is not apparently aphid-borne. In a series of transmission tests using the aphid *M. persicae*, Bawden & Kassanis (15) showed that 105 infections with virus Y developed out of 130 tests, whereas none at all developed out of a similar number of tests using virus C. However, there is some evidence that the vector-relationship may not be quite so clear cut as this. As Bawden & Kassanis point out, odd cases of spread of potato virus C do occur in the field. This may be due to the occurrence of individual aphids with the power to transmit or, what is perhaps more likely, the appearance of variants of virus C which have not lost their vector transmissibility. On the other hand, Bawden & Kassanis state that virus C occurs commonly in old potato varieties and suggest that its existence can be attributed to the vegetative propagation of these old varieties without which it might well be extinct. They think this may mean that in the past the virus was more readily transmitted and that another insect vector was involved. Alternatively, it might mean that prolonged vegetative propagation has lessened its transmissibility by aphids.

A paper just published by Watson (16) is highly relevant to this problem. She finds that a strain of potato virus C has undergone some intrinsic change that makes it aphid-transmissible. Furthermore, this change is apparently reversible and is associated with passage through the Majestic variety of potato. Watson considers that the fact that aphid-transmissibility was often lost after passing the culture through potato suggests that the change was probably a qualitative one, the virus having mutated from the C^p to the C^n strain while infecting *N. glutinosa*, the reverse mutation being induced by reinoculation to potato.

This seems to be the only virus for which a reversible change in aphid-transmissibility has been experimentally demonstrated. It is very interesting to compare this with the production of host-induced, reversible changes in a strain of T.M.V. [Bawden, (9)]. Hollings (17) has found that strains of

chrysanthemum (tomato) aspermy virus appear to differ in their aphid relationships. He cites the case of one isolate that, at first readily transmitted by aphids, became very difficult to spread by this means after two years' continuous transmission to tobacco by mechanical inoculation.

The complex virus disease of tobacco known as rosette [Smith (18)] presents an interesting study in insect-virus relationships. The rosette disease is caused by the united action of two components known respectively as the "vein-distorting" and the "mottle" viruses, only the latter being mechanically transmissible. While these two are together in the plant they are transmitted with great efficiency by the aphid *M. persicae*, both being of the persistent type, i.e., they are retained by the vector for long periods without further access to a source of virus. However, if the two viruses are separated, then the mottle virus is no longer aphid-borne; it is essential for the two to be together in the plant for the mottle virus to be aphid-transmitted. The presence of the vein-distorting virus in the aphid prior to feeding on the mottle-infected plant has no effect. It is not easy to find an explanation for this phenomenon since the obvious one of greater concentration of mottle virus in the presence of the vein-distorting virus does not seem to be the answer. It may be that we have here another example of loss, or partial loss, of vector-transmissibility on the part of the mottle virus, but it is difficult to see why the presence of the vein-distorting virus is necessary to restore the insect relationship. It is apparently only the vein-distorting virus which can do this, since other somewhat similar distorting viruses have no such effect when mixed in the plant with the mottle virus.

Changes in host range and symptomatology.—Mention has been made earlier of the two viruses of tomato bushy stunt and tomato black ring, both of which were originally described by the writer. The first-named virus made only a brief appearance, being recorded from one or two tomato-growing centres. It then disappeared from commerically grown tomatoes and was only once recorded in Britain between 1935 and the present time. It has now appeared in Italy where it was found in 1955 affecting tomato plants [Gigante (2)]. In 1956, through the courtesy of Mr. O. Lovisolo, the writer received some petunia plants from Italy infected with a severe mosaic disease. Inoculation tests on *Datura stramonium*, *V. sinensis* and *N. glutinosa*, together with examination of the purified virus, showed it to be that of tomato bushy stunt. The strain of virus from petunia differed, however, from the type strain in being apparently unable to infect tomato. Two lots of 50 plants each, var. Kondine Red, were inoculated, but none became systemically infected, and only a few local lesions developed on one or two of the plants. It should also be pointed out in this connection that the type strain, after years of propagation in *Datura*, seems also to be losing its ability to infect tomatoes. During the summer of 1956 a number of lettuce plants were received apparently infected with a virus disease. Inoculation from these plants to tobacco resulted in the production of an extremely severe ringspot disease, the rings themselves being unusually pronounced and forming

bizarre patterns. It subsequently transpired, however, that the symptoms on the lettuce did not seem to be connected with the ringspot virus, which does not cause any symptoms in the lettuce plant. At first this virus was given the name of lettuce ringspot, but further experiments suggested a similarity in symptoms to those caused by tomato black ring virus first recorded some ten years earlier, but not seen personally by the writer since then. Various tests proved that the lettuce virus was a strain of tomato black ring, differing slightly, however, in some of its reactions on various hosts. On tomato during July the lettuce virus produced a reddish veinal necrosis and sometimes a lethal stem necrosis. Later in the season the symptoms took the form of a rather necrotic ring and line pattern; in no case were the black rings observed from which the type virus takes its name. In both viruses, however, the characteristic recovery from all visible symptoms ensued, but virus was still present in the recovered tissues. Another symptom variation was observed on *Chenopodium amaranticolor*; the local lesions produced on this test plant by tomato black ring virus consisted of clear, single necrotic ringspots whereas those produced by the virus from lettuce were small necrotic spots, without ring formation.

It is fairly clear that a mutation has occurred in the tomato black ring virus after its natural transfer to lettuce, always supposing that the tomato virus was the type strain. Incidentally, the present culture of the tomato black ring virus at Cambridge does not appear to infect lettuce. Its mode of transfer to lettuce has not been discovered; it is possible that the virus is soil-transmitted by analogy with the ringspot virus causing raspberry leaf-curl in Scotland [Harrison (19)], and this possibility is being investigated. One of the more unusual symptoms characteristic of both the tomato and lettuce virus strain is the production of large enations on the underside of the hothouse type of cucumber, var. Telegraph. These enations are identical in appearance with those produced by squash mosaic virus on squash [Freitag (20)] and since the squash mosaic virus is beetle-transmitted, this may be the case also with tomato black ring. However, this has not yet been proved.

Some latent viruses.—In 1930 Salaman & Le Pelley (21) discovered a latent virus present in all stocks of the potato variety King Edward to which they gave the name "paracrinkle." This virus had no apparent means of natural spread and was never found in any other plant or different potato variety. It seemed to be transmissible only by grafting, and it has come to be regarded as one of the major puzzles of the study of plant viruses; it is often quoted as good circumstantial evidence for the heterogenesis of viruses. Later, Bawden, Kassanis, & Nixon (22) showed that, contrary to previous ideas, the virus was transmissible to some plant hosts by mechanical inoculation. However, the problem of its natural transmission to the original King Edward seedling still remained unsolved. In 1954 another virus latent in several potato varieties, including King Edward, was discovered [Rozendaal (23)] and named potato virus S. This virus is easily transmissible by mechanical means but not, apparently, by aphids. Associated with these two is a

third latent virus which was found in *Dianthus barbatus* (Sweet William) and in *D. caryophyllus*, (carnation) [Kassanis (24)]. Known as the carnation latent virus, it also occurs in several potato varieties, notably King Edward, and is aphid-borne, the vector being *M. persicae* Sulz.

We have the interesting phenomenon of no less than three latent viruses occurring in the same potato variety, two of which appear to have no natural means of spread, the third being aphid-transmitted. In a recent paper Kassanis (25) has shown that all three of these viruses are related serologically to each other. This suggests an answer to the puzzle of the paracrinkle virus. Presumably both this virus and potato virus S are mutants from a common stock of which the carnation latent virus might be the type. Since the latter has a wide host range and is readily carried by the aphid *M. persicae* which itself feeds on a great variety of plants, it is easy to explain the wide occurrence of the carnation latent virus in the King Edward potato. The next assumption is that the carnation latent virus mutated producing strains without its insect-transmissibility. This tendency would, of course, be heightened by the continued vegetative propagation of the potato. In a later section we discuss what appears to be precisely this phenomenon which has taken place in a virus-infected plant of *A. hirsuta* (rock cress) kept under observation for a period of 21 years.

The other latent virus to be briefly discussed is that present in sugar beet and its varieties, the mangold, red beet, etc. Although it resembles the paracrinkle virus in some respects, it differs in the fact that it does spread by some natural means. The virus is much more easily transmitted by mechanical inoculation than that of paracrinkle, but it is very unstable and rapidly loses infectivity. For this reason it seems unlikely that it is transmitted by the soil and all seed-transmission tests have proved negative [Smith (26)]. The most likely explanation is the existence of some arthropod vector which has not been identified although, here again, there are some anomalies which militate against such an explanation. For example, the virus never seems to be systemic in the sugar beet: while inoculation from one leaf may give a positive reaction, inoculation from another leaf of the same plant may be negative. Furthermore, the virus content of an infected sugar beet falls continuously so that towards autumn no more virus can be detected in the plant.

Arabis Mosaic Virus: a Study in Mutation

Since it does not fall to the lot of every plant virologist to keep the same virus-affected plant under observation in a rather unnatural environment over a period of 21 years, the following notes may be of interest. During the summer of 1935 a plant of *A. hirsuta* was found infected with the virus of cabbage black ringspot but no other virus could be detected in the plant at the time. The plant was put into the virus collection at Cambridge and kept there in the insect-proof glasshouse. In December, 1940, during a spell of low temperature, occasion arose for tests to be made from the *Arabis* plant which

yielded only the cabbage black ringspot virus. Some weeks later it was noticed that the leaves of the plant now showed a type of symptom differing from those due to the ringspot virus. Inoculation to a series of test plants revealed the presence of a second virus. This virus was studied by Smith & Markham (5). The virus was inactivated by a 10 min. exposure to a temperature of 60°C. but not by a similar exposure at 50°C. The dilution end-point in crude-expressed sap from White Burley tobacco plants was between 1:100 and 1:1000 and the longevity of the virus in infective sap was between 48 and 72 hr. at room temperatures. The virus was sap-transmissible but was not very infectious, and all attempts to transmit the virus by means of the aphids *M. persicae* and *Aphis fabae* were negative.

On tobacco the symptoms produced by this virus were extremely characteristic. As a rule there were no definite local lesions, although chlorotic spots on the inoculated leaves were occasionally observed. Systemic infection took the form of scattered chlorotic rings, sometimes concentric, on the outer leaves. The central shoot then developed a preliminary pallor or chlorosis followed by a very characteristic splitting of the leaves, and one or both sides of the lamina were stripped away. These leaves invariably became necrotic. This peculiar stripping or shredding of the central leaves, together with a reddish necrosis, was the most characteristic symptom of the whole disease. As the central leaves developed, the necrosis increased and the tips puckered up and bent downwards and inwards. At the same time necrotic spots developed on the rest of the plant with a tendency to become ringlike.

The virus was also transmissible to cucumber, on which it produced a systemic mottle not unlike that of cucumber mosaic virus itself, but the incubation period in the plant was rather long. *P. vulgaris*, the French or string bean, was susceptible to infection and developed a systemic disease which first showed as small yellow flecks on the young leaves, about a week or ten days after inoculation. There were sometimes raised blisters on the leaves with the intervening areas pale. The yellow flecks increased in size producing a fairly bright mosaic mottle; this was followed by a severe necrosis resulting in the death of the growing points of the youngest leaves. It may be worth mentioning here that great difficulty was experienced in transmitting the virus to healthy *Arabis* seedlings.

Some of the properties of the virus and the disease produced in cucumber suggested that the original *Arabis* plant might have been accidentally infected with one or other of the cucumber mosaic virus strains in the collection at Cambridge. The *Arabis* virus differed, however, from cucumber mosaic virus in the following properties: it would not infect *D. stramonium* which is a susceptible host; there was no cross-immunity between it and a yellow strain of cucumber mosaic virus; and it was not aphid-borne.

During the next seven years the original plant of *A. hirsuta* was kept going in the glass house by vegetative propagation. In 1951 Dr. D. O. Norris, who was working in the writer's laboratory at the time, undertook a further examination of the *Arabis* plant which had now been propagated by means of

cuttings for 16 years. He found the cabbage black ringspot virus unchanged and also the *Arabis* mosaic. However, by picking out local lesions developing on *N. glutinosa* after inoculation with the latter virus, he found another virus which differed in its properties from the first *Arabis* mosaic virus. On cucumber it produced a mottle with yellow rings instead of a mosaic, and on *N. glutinosa* it gave ring-like local lesions followed by a systemic mottle. This virus, unlike the preceding one, was transmissible to *D. stramonium*, on which it produced a ring-like mottle. The virus was further studied a little later by M. E. Short who transmitted it to *P. vulgaris* which developed a necrotic mottle with necrosis of the growing point. All attempts to transmit the virus by means of aphids were negative. The physical properties were somewhat similar; the dilution end- point was 10^{-2}; the longevity was four days; and the thermal inactivation point was between 50° and 60°C.

In 1956, Dr. I. Harpaz undertook a further study of the *Arabis* plant, which had by this time been propagated vegetatively for 21 years, and he has kindly allowed some of his unpublished results to be quoted here. He found the cabbage black ringspot virus still unchanged but the mosaic virus appeared to have altered once more in certain characteristics. As to the physical properties the change does not seem very marked. The thermal inactivation point was 59°C., the dilution end-point seemed somewhat higher as infections were obtained at 1:1000 but not at 1:2000. The longevity was 72 hr. at room temperature and the sap was still infective following a 24 hr. incubation at 37°C., a property which sharply differentiates the virus from that of the various strains of cucumber mosaic virus, all of which are inactivated by this process.

The host range and symptomatology had changed considerably and the virus now failed consistently to infect either *D. stramonium* or *N. glutinosa*. On tobacco, vars. White Burley and Kawala, the symptoms consisted of a faint mottling similar to that caused by cucumber mosaic virus. This mottling invariably faded under both winter and summer conditions. On cucumber a mosaic was produced which was indistinguishable from that caused by cucumber mosaic virus itself, although the systemic disease resulted in bud proliferation and shortening of the internodes. Large local lesions, consisting of a maroon ring with a white centre, developed on the inoculated leaves of *Gomphrena globosa* without systemic infection. This is rather similar to the behaviour of cucumber mosaic virus on the same host plant. All attempts to transmit the virus by means of aphids were unsuccessful.

Unless we are going to accept the principle of heterogenesis in plant viruses, the only other explanation of the foregoing phenomena seems to lie in a continual process of mutation on the part of the original *Arabis* mosaic virus. If we assume that the *Arabis* plant was infected in the first place by an aphid-transmitted strain of cucumber mosaic virus, it may be of interest to compare what properties the three apparent mutants have in common with CMV and in what respects they differ. *Mutant No. 1* resembles CMV in the

mosaic disease it produces on cucumber and in some of its physical properties. It differs in host range and symptomatology; it does not infect *D. stramonium;* it produces quite unusual symptoms on tobacco; and it invades systemically *P. vulgaris.* More important it is not aphid-transmitted and shows no cross-immunity with a yellow strain of CMV. *Mutant No. 2* was somewhat similar in physical properties but the disease produced on cucumber differed in being a mottle with yellow rings. On *N. glutinosa* ring-like local lesions followed by a systemic mottle developed. On *P. vulgaris* the virus gave rise to a necrotic mottle with necrosis of the growing point. Unlike Mutant No. 1, the second virus was transmissible to *D. stramonium;* it was not aphid-borne. *Mutant No. 3* showed slight differences in its physical properties and also in its host range. It failed to infect either *D. stramonium* or *N. glutinosa* and on tobacco it produced only a mild mosaic which soon faded out. It was not aphid-transmitted.

If we accept that the viruses, other than that of cabbage black ring, are all derived from a chance infection with a strain of CMV, then by far the most interesting development is the loss of insect-transmissibility. This would account for the appearance in isolated and possibly unusual host plants of viruses which seem to have no natural means of spread. If this should prove correct it would rather alter our conception of plant virus relationships with insect vectors, and suggest that the vector relationship is a much less constant factor than has hitherto been supposed.

Discussion and Summary

With advancing knowledge of the subject, it becomes increasingly clear that certain properties of viruses which have for long been regarded as unalterable characteristics are in reality not so unchangeable.

One of these is the vector-relationship of a virus which hitherto has been considered a fixed property. Apparently this is not so and, as we have seen, a virus kept for long periods out of contact with its insect vector is liable to lose its insect-transmissibility. A second property of viruses to which our attitude needs orientation is that of mutation. It has long been known, of course, that mutation does occur, notably in tobacco mosaic virus in which a "yellow" or a "white" mutant frequently develops. Several such related related viruses have been mentioned earlier. What is new, however, is the conception of a much greater frequency of mutation, together with alterations in properties hitherto regarded as fundamental. A change of attitude is also needed towards a third characteristic of plant viruses, i.e., mode of transmission. Although it has been known for some years that the vector of the reversion disease of black currants was a mite, this type of arthropod vector does not seem to have received any serious consideration until just recently when no less than three entirely unrelated viruses have been found to be transmitted by Eriophyid mites. These are the viruses of wheat streak mosaic, fig mosaic, and peach mosaic.

It has been customary to think that soil-transmission plays a very small

part in the spread of viruses and here, again, it seems possible that this method of virus transfer is very much more common than is generally supposed. For example, Harrison (19) found three soil-borne viruses in soil collected round raspberry plants infected with leaf-curl. Two of these were of the ringspot type and the third appeared to be one of the tobacco necrosis viruses. If we change our conception of these various properties, then some plant virus problems become less insoluble. The classical puzzle of the paracrinkle virus in King Edward potatoes is solved by assuming that it was a mutant from the aphid-borne carnation latent virus which lost its insect-transmissibility after long vegetative propagation while retaining its characteristic of latency. By the same reasoning, potato virus S could be another mutant from the common stock. Such an explanation is supported by the behaviour of the virus in *A. hirsuta* which, as we have shown, has apparently undergone somewhat similar changes during 21 years of actual observation.

We are still, however, without an answer to some of the problems mentioned in this article. The sudden appearance of a virus in a tobacco plant growing in an insect-proof glasshouse, and for which no insect vector could be found as in the case of tobacco broken ringspot, still requires an explanation. We can, of course, suppose that the virus was very occasionally seed-transmitted and this happened to be one of the occasions. The various examples of virus diseases developing in single isolated plants out-of-doors with no apparent means of spread, such as the lovage mosaic, and a curious virus found in the weed *Pimpinella saxifraga*, could possibly be explained on the grounds of mutation or occasional seed transmission. Since such viruses do not seem to spread in nature, the existence of a hypothetical vector need not be assumed.

The presence of the latent virus in sugar beet cannot be explained by any of the above possibilities. This virus does apparently spread with great readiness and the solution to the problem will probably be found in the mode of spread. If it is seed-borne then it must be in quantities too small to be detected by methods at present available and the most likely explanation seems to lie in an undetected vector of an unusual type.

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SOIL MICROBIOLOGY[1]

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The field of Soil Microbiology was last reviewed in this journal by Lochhead in 1952. The present review is thus intended to deal with the period from 1952 to 1956 inclusive, although a few papers published before this period have been included for various reasons. A complete coverage of the literature is not possible in a review of this length, and where a subject has been recently reviewed the authors have only mentioned the subject briefly and have quoted the review as a source of references.

TECHNIQUE

Soil microbiologists have been concerned for a long time with the problem of studying the soil micropopulation in the natural solid soil as little affected by manipulation as possible. A method for demonstrating microorganisms in sections of soil has been developed by Alexander and Jackson. A core sample stained in bulk with cotton blue is vacuum-impregnated with a polyester resin and sectioned by a method similar to that used for rock specimens. This method is very promising, particularly for the observation of fungal hyphae (1).

The method initiated by Rossi and Cholodny, in which the growth of microorganisms is observed on slides buried in soil, is used in its original form as well as in various modifications (60, 182). It has always given results difficult to interpret in terms of microbial habit in the soil mass itself; but useful information has been obtained on antibiosis by modified Rossi-Cholodny methods. Dobbs & Hinson have used one to study fungistasis (47); Chinn, and also Stevenson, have placed fungal spores on slides and inserted them in soil. By subsequent examination of the preparations the percentage germination can be estimated, while by pregerminating the spores, deformed growth of the hyphae, or lysis, can be observed (35, 199). Methods of isolating *Acrasieae* from soil, by using a suspension of edible bacteria, either sprinkled on soil, or in agar, have been developed by Kitzke (105) and by Borg (19).

Most methods used for the study of soil microorganisms, however, still depend on making a suspension of the soil. In the most direct application of this method, films are made from the suspensions, and the organisms in them examined microscopically. Tchan has used fluorescence microscopy for observing and counting algae, and Tchan & Bunt have developed a method in which protozoa, fixed in the wet film with osmic acid or formalin vapour, are then stained with erythrosin and methyl green (208, 213).

[1] The survey of the literature pertaining to this review was concluded in December, 1956.

Experiments made to test the agreement between estimates of the numbers of microorganisms in replicate samples of soil, usually based on plate counts, have given very different results in different localities and soil conditions. In contrast with the good agreement obtained in past work on the very long term arable experiments of Rothamsted, Rose & Miller found very great variation in plate counts of fungi from replicate soil cores taken from virgin land and pasture in New Zealand, and have proposed a method for reducing error by bulking numerous cores (176).

In some cases plate counts of bacteria made at a range of dilutions give estimates of numbers per gram that rise with the degree of dilution. Lavergne & Augier attribute this to the retention of bacteria by the internal surfaces of the pipettes used in making and distributing the dilutions, and also to the break-up of clumps of bacteria (112). The same effect would, of course, be observed if the soil contained some factor inhibiting bacterial growth on agar, which was progressively removed by increasing dilution of the soil suspension (138). Miller and his colleagues recommend the use of oxgall as a bacterial inhibitor in agar media for isolating fungi and yeasts, as it can be sterilized in the medium (11, 143), and de Barjac proposes the use of soil humic acid to acidify media used to grow organisms from acid soils (41).

Several methods based on fractionation of soil suspensions have been used for the isolation of fungi (4, 113, 234); Chesters & Thornton have compared six different techniques for isolating soil fungi, and find that two of them, dilution plates and Warcup's soil plates, favour species which sporulate abundantly. The greatest variety of species was obtained by the screened immersion plate method of Thornton (34, 235).

Among techniques for estimating the total microbial activity in a soil, the macrorespirometer of Swaby & Passey may be mentioned (205). Pochon and his colleagues measure various types of microbial activity by comparing the rates at which a reaction takes place in media inoculated with a series of dilutions from a soil suspension (43). Tchan has proposed that nutrients should be assessed by measuring the growth of the indigenous algal flora of a soil with and without added test compounds (211).

EFFECTS OF SOIL CONDITIONS ON THE MICROFLORA

Locality and Type

Bacteria.—It is usually supposed that all species of soil bacteria have a world-wide distribution; Prévot & Moureau have shown that this is probably true of the common anaerobic bacteria, which they have isolated from a variety of soils, including some from the Antarctic (169). Curiously enough, thermophilic bacteria have been found by McBee & McBee, in an Arctic soil which is frozen for most of the year (128).

A relation between the quality of the bacterial flora of a soil and its fertility was found by Pochon & Coppier, who compared neighbouring fertile and infertile soils from two localities in France. The fertile soils contained more microorganisms in general, but fewer cellulose decomposers than in-

fertile soils; and non-symbiotic nitrogen fixers were found in the fertile, but not in the infertile soils (164). On the Broadbalk wheat field at Rothamsted, on the other hand, Skinner, Jones, & Mollison found larger numbers of bacteria, actinomycetes, and fungi in plot 2B, given farmyard manure, than in plot 7, given artificials, or plot 3, unmanured; but there was little difference in numbers between these last two plots, which differ greatly in their yield of wheat (190). Pochon and his colleagues have shown that a compost may contain a high total number of bacteria developing on agar plates, and yet be very poor in nitrifiers, nitrogen fixers, and other important groups (166).

The most striking effects of soil amelioration on the microflora are shown by reclaimed peaty soils which, like acid forest soils, are very poor in microorganisms in their natural state. De Barjac has made a detailed study of the microflora of several types of peat before and after improvement (42). Boquel, Kauffmann, & Toussaint, in a study of the tropical forest soils of the Ivory Coast, found increased numbers of nitrogen-fixing clostridia, and of cellulose-decomposers, during the rainy season. Clearing the forest decreased the numbers of the latter (18).

Fungi.—Peyronel represents the character of the fungus flora of a particular soil by dividing the species into 8 groups, and showing the frequency of each group on a compass diagram. He has compared the flora of different temperate and tropical soils by this method (163). Stenton has reported on the fungus flora of the soil of Wicken Fen (195), and Gordon on the occurrence of *Fusarium* species on cereal plots in Canada (62). Zeidberg & Ajello report on the presence of two pathogenic fungi in Tennessee soils (248). Forest and agricultural soils were compared by Welvaert & Veldeman, who found a greater variety of fungi in polder soil (pH 7.5) than in a sandy loam forest soil (pH 4.3). They suggested that, in the latter, the antagonistic action of *Trichoderma* and *Penicillium* might be an important factor (239). The short time needed for trees to affect the microflora is illustrated by work reported by Meyer, who found differences in the fungal population under pasture and under tree saplings (141). An influence of the type of tree growing on forest soils is suggested by the results obtained by Chase & Baker, who showed that the ratio of fungi to bacteria and actinomycetes was higher under conifers than under maples (33).

The soil yeasts are a little-known group that has been studied recently by Capriotti (32) in Holland and by Di Menna (46) in New Zealand. Those found in New Zealand soil included the pathogen *Candida albicans.*

Vertical Distribution of Microorganisms

The distribution of microorganisms down a sandy soil profile is reported in a paper by Blue, Eno, & Westgate. Highest numbers occurred in the top six inches. Very few microorganisms were found in the A2 horizon, nine to twelve in. down, which was deficient in nutrients. More were found between 12 and 30 inches below the surface; numbers at this level were increased by

dressings of potassium and of nitrate (15). Tchan & Whitehouse have shown that algae are confined to the top few mm. in wet soil, and that there is no evidence that they can live under the soil surface as heterotrophs (214). Garbosky reports that *Azotobacter* is found at depths down to two metres in Argentine soils, and that it may be more abundant between 50 and 100 cm. down than at the surface (58). The distribution of fungal species at different levels in sandy podsol profiles was studied by Jeffreys and co-workers, who found that plate counts decreased generally with depth but showed a secondary maximum in the B horizon, noticeable for the preponderance of *Mucor* at this level (92). Guillemat & Montegut observed, in plots from the long-term field experiments at Grignon (69), that different species of fungi occurred at different depths. Burges & Fenton conclude that the ability of fungi to live deep in the soil is a consequence, not, as is commonly supposed, of their ability to do without oxygen, but of their ability to withstand high concentrations of carbon dioxide (25).

Nutrients

Starkey has reviewed the whole subject of the need of different microorganisms for inorganic nutrients (193). Jensen has shown that *Azotobacter* needs much more magnesium than the related genus *Beijerinckia*, which, unlike *Azotobacter*, cannot partly replace its need for molybdenum by using vanadium (95, 96). Shug *et al.* have thrown more light on the reason why some microorganisms need molybdenum, by showing that it is a cofactor for the hydrogenase of *Clostridium pasteurianum* (186).

Lochhead & Thexton found that an important group of soil bacteria required vitamin B_{12}, replaceable by an aqueous extract of soil (125). More than a quarter of the 500 isolates from soil studied by Lochhead & Burton were found to require one or more of the following growth factors: thiamin, biotin, B_{12}, pantothenic acid, folic acid, nicotinic acid, and riboflavin (122). In soil, the growth factor requirements of such bacteria can, no doubt, be supplied by other organisms; for example, Burton & Lochhead found that several species, especially of *Rhizobium*, can synthesise vitamin B_{12} (27). Soil extract has also been found by Lochhead & Burton to contain at least one growth factor other than B_{12}, and not supplied by yeast extract. Culture filtrates from a number of soil bacteria having simple nutrient requirements were able to supply a growth factor having the same effect. One of them, named *Arthrobacter pascens*, synthesises such a factor, to which a related species, *Arthrobacter terregens*, is exacting. Soil extract is therefore useful in the study of soil bacteria, not only as a source of minerals, but also of some organic nutrients (123).

Partial Sterilisation

In their early work on partial sterilisation, Russell & Hutchinson suggested that increased bacterial activity following partial sterilisation was due to the destruction of soil protozoa. Singh & Crump used the improved

dilution technique, developed by the former (Singh), to estimate numbers of amoebae in soils from forest nursery beds, untreated and partially sterilised with steam and formalin. Bacterial counts were also made by plating. In the formalin-treated soil, bacterial numbers rose above those in the untreated, and numbers of amoebae were reduced. But in steamed soil, numbers of both bacteria and amoebae increased over those in the untreated soil. These effects were found to persist over a period of six months (187). It is thus unsafe to generalise as to the effects of different types of partial sterilisation on relative numbers of amoebae and bacteria. Stout's qualitative observations of steamed glasshouse soil also showed that a number of species of protozoa survived the treatment (202). The fungal population seems to be more drastically affected by partial sterilisation of soil. Mollison followed the changes in fungi in the same forest nursery plots that were examined by Singh and Crump. Both steam and formalin treatments almost completely eliminated the fungi and, even after later recolonisation, the plate "counts" of fungi were much reduced, an effect which lasted for the 25 months during which counts were made. The fungal population was still varied after steam treatment, but after formalin, *Trichoderma viride* appeared to be the principal species that recolonised the soil (146). This fungus is exceptionally tolerant of formalin. Wensley, in a paper giving useful data on the immediate microbiological effects of soil treatment with several fumigants, reports that after treatment with methyl bromide, soil was recolonised in 4 weeks, mainly by *Aspergillus* (240). Evans also followed the recolonisation of soil by fungi after partial sterilisation, in this case with formalin and chlorpicrin (53). Warcup showed that *Pythium* in forest nursery soils is killed by steam or formalin (233). There is also a change in the relative proportions of different groups of bacteria in soil following partial sterilisation. Thus Davies & Owen found that ammonia accumulates in steamed glasshouse soils, because the nitrifying bacteria are killed (39); and Holding found an increase in the percentage of Gram-negative bacteria after steaming. This group also increased after the addition of fresh organic matter or the growth of oat seedlings, suggesting that the effect of steaming may be partly due to release of nutrients which favour these bacteria (84).

An interesting specific effect was noted by Bromfield in some experiments with soil treated with carbon tetrachloride. After treatment these soils evolved hydrogen sulphide from ammonium sulphate added to them, apparently owing to the activity of *Bacillus megaterium*, which, when isolated from the soil, would carry out this reduction in pure culture. The action of carbon tetrachloride was to eliminate certain bacteria which prevent this reducing activity of *B. megaterium* from taking place in untreated soil (21). The practical aspects of disinfestation of soil by heat, flooding and fumigation have recently been reviewed by Newhall (151).

Weedkillers and other poisons.—More and more very poisonous substances are being added to soils all over the world to kill weeds or insects, and it is fortunate that most of them have been found, on investigation,

to have no lasting harmful effect on useful soil microorganisms. Magee & Colmer (129), for instance, found that *Azotobacter* was poisoned in culture only by much larger doses of herbicides than would ever be used in practice, and the same was found to be true of four commonly-used insecticides by Callao & Montoya (31), who worked with soil as well as liquid cultures. Gray has carried out a series of studies on the effects of hexachlorocyclohexane and its *gamma* isomer (Gammexane), and finds that the effect of both isomers is very much less in soil than in culture media; but it did reduce the count on soil-extract agar plates (66). Staněk (191) reports that Gammexane actually stimulates *Azotobacter*, however, and Koike & Gainey (108) and Jones (101) find that the total plate count of bacteria is increased by large doses of 2-4-D, CADE and DDT; and none of these three compounds affected nitrification in any dose likely to be used in practice. Surface-active agents are regularly added as spreaders to agricultural sprays; Ivarson & Pramer have found that Tween 80, a non-ionic compound, was rapidly decomposed in soil, and had little or no undesirable biological effect. The cationic spreader Ceepryn was more slowly decomposed, and in large doses it reduced the total plate count and hindered nitrification. An anionic compound, Nacconol NRSF, on the other hand, was not decomposed at all, and had serious and lasting toxic effects (89). The same may be true of radioactive phosphorus, and Goring & Clark recommend that it should not be used for experiments on plants if the soil in which the plant is growing is meant to be in its natural state (63).

ACTIVITIES OF SOIL MICROORGANISMS

Soil Structure

There is evidence that some fungi may hold the soil together by the network of their mycelium; Downs, McCalla, & Haskins found that one out of twelve cellulose-decomposing fungi improved the structure of a soil poor in organic matter (50). The gum produced by *Agrobacterium radiobacter* improved aggregation (173); Rorem suggests that bacterial gums, besides increasing aggregation, may be important to the species which produce them as a mechanism whereby they can concentrate ions that they need out of the surrounding soil (175). Hely & Bonnier found that synthetic soil conditioners increased the numbers of bacteria which produced natural gums, and so had a double effect on soil structure; the synthetic compounds were not toxic (76). Mortensen & Martin found that two synthetic soil conditioners were not toxic to microorganisms, but were very resistant to decomposition (147). In certain soils the addition of synthetic aggregating substances has been found to improve nodulation of soy beans and of lucerne (185, 78).

Breakdown of Natural Carbon Compounds

Not much interest is being taken at present in this aspect of Soil Microbiology. Reese & Levinson have compared the breakdown of cellulose by different species; Kox found that aerobic bacteria, as well as fungi, are responsi-

ble for the breakdown of cellulose and pectin in Sphagnum peat, and McBee describes a thermophilic anaerobe which can decompose cellulose (110, 127, 172).

Until recently, fungi were thought to be the only organisms capable of decomposing lignin; but Fischer, Bizzini, Raynaud, & Prévot have found that bacteria which can break down lignin are very widespread, and especially numerous in forest soils. All their strains were species of *Pseudomonas*, and most of them could attack benzoate and other aromatic carbon compounds as well as lignin (55). Henderson & Farmer found that some soil fungi could utilize aromatic compounds such as syringaldehyde, which might be breakdown products of lignin (79). Veldkamp found that a number of soil microorganisms can decompose chitin; actinomycetes seem to be the most numerous. Twenty-three different actinomycete species were found to be chitin-decomposers, and likewise 50 species of bacteria, including the myxobacterium *Cytophaga johnsonae*. Ammonia and acetic acid are the final breakdown products in cultures of this species and of a new species, *Pseudomonas chitinovorans* (220). The breakdown of pectin in fallen leaves has been studied by Wieringa; he finds that the first agent of breakdown is always the fungus *Pullularia pullulans*, which is present on the surface of leaves while they are still on the tree. Pectin-decomposers in acid soils are mostly fungi, but in well-limed soils, actinomycetes predominate (242).

The Nitrogen Cycle

Soil microbiologists have written over a hundred papers on this subject since 1951, but their interest is very unevenly distributed; over three-quarters are concerned with the fashionable subjects of *Azotobacter* and nitrification. There is practically no work on the formation of ammonia, which is overdue for investigation by modern methods. It is usually supposed that the breakdown of proteins is the source of soil ammonia; but Veldkamp has recently shown that chitin, which is abundant in soil in fungus hyphal walls and arthropod integuments, is an important ammonia source (220). He incubated chitin in soil, and found that up to 60 per cent of the nitrogen in it was recovered as nitrate, presumably formed by the oxidation of ammonia. It is also possible that nitrogen fixers supply ammonia directly to the soil (as Winogradsky thought), for Delwiche & Wijler found that most of the nitrogen fixed in their experiments could be accounted for as nitrate (44).

Nitrification.—There have been recent reviews on this subject by Lees (116, 117) and Meiklejohn (132, 134). It is becoming increasingly apparent that the agents of nitrification are the classical autotrophic nitrifiers of Warington and Winogradsky, which are ubiquitous, and which convert ammonia quantitatively to nitrite and then to nitrate. There are several recent reports of heterotrophic microorganisms which form nitrite or nitrate by oxidation; but, if the amounts are stated, they are always very small (54, 56, 85, 88). The very laborious dilution method is still the only satis-

factory way of counting nitrifiers in soil (37). Many of the colonies which develop on Winogradsky's silica-gel plates are not nitrifiers, and Millbank found that a method with agar disks, similar to Singh's method for protozoa, would not work because agar and soil together were toxic to *Nitrosomonas* (59, 86, 142). It is possible to compare the nitrifying activity of different soils by measuring the rate of nitrification in enrichment cultures, or by the Lees & Quastel percolation method, if the soil structure is good enough (133, 200). This method was used by Stevenson & Chase, who found that nitrification was less under a grass cover than under bare fallow (200). Mills (144) has also found that different African grass species depress the formation of nitrate to different degrees. In the Uganda soil that he studied, there was an accumulation of nitrate in the top 6 inches in the dry season, though the soil was acid. Meiklejohn found that this soil contained autotrophic nitrifying bacteria (133). Jacquemin & Berlier found that there were very few nitrifiers in a forest soil from the Ivory Coast, and many more in cleared land (90). Burning the vegetation over a Kenya soil was found by Meiklejohn to kill the nitrifiers (136).

There has not been much work on the physiology of the nitrifying bacteria, mainly because of the enormous difficulty of getting them to grow in pure culture. This may be partly due to their need for some growth factor which they can obtain readily in soil, and which is lacking in culture media; but Gundersen (70) tried various vitamins of the B group on pure cultures of *Nitrosomonas*, and Meiklejohn (131) tried several other possible stimulants in enrichment cultures, in both cases with entirely negative results. Though Gundersen (70) found that several amino-acids are very toxic to *Nitrosomonas*, and though Jensen & Sørensen (100) found that the same was true of some organic sulphur compounds, there does not seem to be any truth in the belief that organic matter as such has a mysterious toxic effect on nitrification.

Goldberg & Gainey (61) have studied the effect of clay minerals on ammonia oxidation, and find that ammonium ions are more readily oxidized by enrichment cultures if free in solution than if adsorbed on the clay. This is quite contrary to earlier results of Lees & Quastel with soil (see Lees, 116).

Lees (115) has found, by using very dilute solutions, that hydroxylamine is an intermediate product in the oxidation of ammonia by *Nitrosomonas* cells, and Imshenetskiĭ & Ruban (87) have shown that it is oxidized by cell-free autolysates. Hydroxylamine is also formed in the oxidation of pyruvic acid oxime by heterotrophs; further oxidation to nitrite, in these species as well as in *Nitrosomonas*, is blocked by hydrazine (120). It would be interesting to know if chelating agents such as allylthiourea, which Lees (114), and Hofman & Lees (83), found to stop ammonia oxidation at a very low concentration in suspensions, have the same effect in soil. Lees & Simpson find that the oxidation of nitrite by *Nitrobacter* is interrupted at different stages by chlorate and by cyanate. *Nitrobacter* contains more than one cytochrome,

and they are reduced as nitrite is oxidized (118, 119). In view of these results it is most puzzling that Engel, Krech, & Friederichsen conclude that neither iron nor zinc-containing enzymes are involved in the oxidation of nitrite, though *Nitrobacter* needs iron for growth (52). These workers also investigated the amino acids in *Nitrobacter*, and found the same 18 that Hofman had found in *Nitrosomonas*, and that they themselves had found in *Hyphomicrobium*. The list does not include *alpha-epsilon*-diaminopimelic acid. Hofman found four sugars, galactose, ribose, rhamnose and xylose, but oddly enough, no glucose, in his *Nitrosomonas* preparations (81).

Several workers find that the ratio of nitrogen oxidized to carbon assimilated is higher in old than in young cultures of nitrifiers. Hofman & Lees (82), looking at this from a thermodynamic point of view, think that the ratio increases because *Nitrosomonas* needs more energy to keep increaing concentrations of toxic nitrite out of its cells. But, as Engel *et al.* point out, this can hardly be true of *Nitrobacter*, which forms a less toxic compound, nitrate, from the more toxic nitrite (52). A more probable explanation is given by Bömeke, who thinks that there is a progressive loss of carbon from old cultures, as the cells break down some organic storage material to keep themselves alive (16).

Enrichment cultures have been studied by Klein, who found a way to get rid of *Nitrobacter*, which can be a very troublesome contaminant of *Nitrosomonas*, by supplying ammonia in the form of ammonium borate (106).

Imshenetskiĭ (86) and also Bisset & Grace (12) claim that there are no genera of autotrophic nitrifying bacteria other than *Nitrosomonas* and *Nitrobacter*, and that the genera *Nitrosocystis*, *Nitrosogloea*, *Nitrosospira*, and *Nitrocystis*, described by Winogradsky and his colleagues, are not nitrifiers (86, 12). They base this criticism on the observation that nitrifying cultures may be contaminated with *Myxobacteria*, whose fruiting bodies could have been mistaken for zoogloeal organisms responsible for the nitrification. More definite evidence is needed to substantiate so comprehensive a criticism. Indeed, Palleroni has claimed to have isolated *Nitrosospira* sp. from Antarctic and Argentine desert soils and found that it was a nitrifier (155, 156).

Denitrification.—Wijler & Delwiche, using isotopic nitrogen and soil, showed that denitrification is probably entirely due to microbial action, as all the nitrogen in the reduction products was derived from nitrate, which excludes the formation of nitrogen by a non-biological reaction between nitrite and amino groups. They also found that nitrous oxide, as well as nitrogen gas, was released when soil and nitrate were incubated together (243). The soil they used therefore presumably contained organisms such as the *Denitrobacillus* species studied by Verhoeven, that produce nitrous oxide as well as nitrogen in the reduction of nitrate (221). The effect of aeration on denitrification may vary with the species of bacteria present in a soil, as Marshall *et al.* found that different *Pseudomonas* strains are differently affected by aeration. One strain may only reduce nitrate under conditions of

almost complete anaerobiosis, while another may be so little affected by oxygen that it is almost impossible to stop it reducing nitrate in culture by increasing the air supply (130).

Nitrogen fixation.—There has been a recent experiment with isotopic nitrogen by Delwiche & Wijler, the results of which were almost entirely negative. In the soil that they studied they found negligible fixation in bare fallow, and very little under grass (44). In consequence of this, some microbiologists may conclude that non-symbiotic nitrogen fixation never adds any nitrogen to any soil in any circumstances. But, if one leaves the laboratory and turns to the field, it is obvious that the nitrogen supply is maintained in soils in which legumes are not growing, and in which leaching certainly, and denitrification probably, takes place. The evidence as to non-biological fixation is most contradictory, but recently Bjälfve has shown that light is not an agent of nitrogen fixation (13). Biological fixation has been reviewed by Wilson & Burris (245), by Fogg (57), and again by Wilson (244), who deals especially with the mechanism of fixation.

Azotobacter.—There is a certain irony in the fact that so much is being written about *Azotobacter*, as it is becoming more and more doubtful if this genus is of real practical importance in adding to the soil's nitrogen supply, for there seem to be many soils, in various parts of the world, where it is never found (20, 102, 135). Where it does occur, it is usually found in very small numbers, and not only in poor soils, like the Arno Atoll soils examined by Stevenson (197), for Meiklejohn (137) found less than 2000 *Azotobacter* cells per gram throughout a series of counts on Rothamsted soil. But Pochon and his colleagues record a count on the very fertile Nile silt, in which about 8000 *Azotobacter* per gram of dry matter, as well as nitrogen-fixing clostridia, were present (167). Counting *Azotobacter* in soil is difficult, however; neither Hely & Bonnier (76) nor Meiklejohn (137) were able to obtain consistent results with Winogradsky's method of "plaques moulées"; and when they tried his other method, of crumbs of soil sprinkled on silica gel, they found that a colony of *Azotobacter* developed from every crumb. Eventually they used surface inoculation on nitrogen-poor agar, as did Tchan (207), who also used a dilution method with liquid cultures. Absence or scarcity of *Azotobacter* may be due to lack of moisture, to lack of calcium or phosphate, and perhaps of other nutrients (135, 206). It might even be due to excess of oxygen, as Parker (158) has found that *Azotobacter* fixes nitrogen more efficiently under reduced oxygen tension; but lack of water is an equally probable explanation for the scarcity of *Azotobacter* near the surface of soils in Argentina, described by Garbosky (58).

Inoculation with *Azotobacter* to increase the yield of various crops is regularly practised in Russia, but it does not seem to be uniformly successful (180, 226, 228, 241). Petrenko suggests that many of the negative results may be due to the use of unsuitable cultures; it is well known to all microbiologists who have worked with *Azotobacter* that long-continued maintenance in artificial culture alters its properties, and use of an old stock culture

from a type collection may well lead to confusing results (162). On the other hand, it is possible that some positive results of inoculation are due to fertilizers added with the bacterial culture (9). Bukatsch & Heitzer claim that strains of *Azotobacter* isolated from the rhizospheres of different plant species differ in nitrogen-fixing power; it is unfortunate that they examined one strain only from each species of plant, and still more unfortunate that their experiment on *Azotobacter*-inoculated peas was carried out in open pots, so that infection with *Rhizobium* cannot be excluded as the cause of the fixation observed (24).

Parker has produced evidence that *Azotobacter* fixes nitrogen more efficiently in the presence of other bacteria than in pure culture. His freshly-isolated cultures fixed 18 mg. N per gram sugar decomposed when the *Azotobacter* was still contaminated with a small motile rod; but when this last contaminant was removed, fixation in the pure culture went down to three mg. N per gram sugar, and only gradually improved after several transfers in a nitrogen-poor medium (160).

It is generally supposed that *Azotobacter* does not grow or fix nitrogen at any pH more acid than 6.0, but recently several acid-tolerant *Azotobacter* strains have been found. Jensen (97) has described a new species, *Azotobacter macrocytogenes*, and acid-tolerant varieties of known species have been found by Tchan (209), Döbereiner (48), and Metcalfe (139). A new species, *Azotobacter halophilum*, which will only develop in saline media, has been found in saline soils in Siberia by Blinkov (14).

Beijerinckia.—Jensen has shown that there are good reasons for separating the aerobic acid-resistant nitrogen fixers of tropical soils from *Azotobacter* and placing them in the genus *Beijerinckia*. They differ from *Azotobacter* in morphology (the cells are much smaller), and also in being able to fix nitrogen at pH 3.5, in needing no calcium, and in being unable to use vanadium in place of molybdenum. Azotobacters occur in the tropics in calcareous soils, but tropical soils are commonly acid, and here Beijerinckias, which are efficient but slow nitrogen fixers, replace them (96). Many attempts have been made without success to isolate *Beijerinckia* from temperate-zone and subtropical soils (210). Derx (45) attributed the tropical distribution of this genus to a possible association with some special genera of plants, perhaps legumes which do not form nodules (e.g. *Cassia* spp.), and suggested that *Beijerinckia* is a facultative symbiont which, unlike *Rhizobium*, has not lost the power to fix nitrogen outside the plant. On the other hand, Kluyver & Becking (107) think that *Beijerinckia* may be confined to lateritic soils. There is a recent report of the occurrence of *Beijerinckia* outside the tropics, as Suto has isolated a nitrogen-fixer, which seems from his description to belong to this genus, from an acid volcanic soil at Sendai, Japan (lat. 38°N) (204). Ruinen has found a new habitat; she has discovered large numbers of *Beijerinckia* cells on the leaves of trees and epiphytes in the tropical forests of Indonesia, a fact which may explain the lavish vegetation of the forest on a soil which gives very poor yields of crops when cleared and planted (179).

Clostridium.—Though very little has been written about *Clostridium pasteurianum* (and related species) in recent years, it is quite possible that these anaerobes account for much more nitrogen fixation than does *Azotobacter.* In the first place, they are much more widely distributed than *Azotobacter;* Rybalkina (181) in Russia, Kaila (102) in Finland, Boswell (20) in England, Meiklejohn (135) in East Africa, and Tchan & Beadle (212) in Australia, found them always, or nearly always present in every soil examined. They are also far more numerous; in contrast to the thousand *Azotobacter* cells per gram, *Clostridium* cells number hundreds of thousands (74, 137). Hart found from a hundred thousand to a million per gram of garden soil, nine-tenths of them vegetative cells and one-tenth spores. Numbers in an oakwood soil were somewhat smaller (74).

It has generally been supposed that clostridia were poor fixers of nitrogen, adding only about two to four mg. per gram of sugar decomposed. But Parker (159) has recently shown that, given suitable cultural conditions, a strain of *Clostridium butyricum* can do as well as the best *Azotobacter,* fixing 27 mgm. N per gram sugar. To obtain this level of fixation it is necessary to grow the bacteria in presence of carbon dioxide as well as nitrogen, in absence of carbon monoxide, and to supply them with growth factors. Parker's strain required biotin and para-aminobenzoic acid, and a strain studied by Virtanen & Lundbom (227) required folic acid.

It might be objected that strict anaerobes could not multiply fast enough to be able to fix much nitrogen in the topsoil; but Hart found that his nitrogen-fixing clostridia were able to grow under aerobic conditions if supplied with combined nitrogen. They did not fix nitrogen aerobically; but it is quite possible that clostridia could grow in topsoil if combined nitrogen were present, and then, in local pockets of anaerobiosis, or at times of temporary waterlogging, proceed to fix nitrogen when the original supply was exhausted (74).

Other Nitrogen-fixers.—The blue-green algae are probably the most efficient of all non-symbiotic nitrogen fixers. De & Mandal estimate that, given sufficient phosphate and molybdenum, algae in flooded rice soils can fix as much as 70 lb. nitrogen per acre in six weeks (40). Blue-green algae are also able to fix nitrogen in some symbiotic systems, for Bond & Scott showed that lichens and liverworts can fix nitrogen if *Nostoc* is present as a partner in them (17). Douin showed that the nodules on the roots of Cycads contained a species of *Anabaena* (49). There is also an increasing list of organisms which can only fix very small quantities of nitrogen, in many cases so small that fixation can only be detected by the use of isotopic nitrogen. Metcalfe *et al.* (140) used this method to find that two yeasts, isolated by the percolation method from acid health soils, were able to fix nitrogen. Anderson (3) describes another poor nitrogen fixer which is apparently a *Pseudomonas,* and Brown (23) has found two nitrogen-fixing *Nocardia,* one of which could decompose cellulose. Newton & Wilson (152) report that the purple sulphur bacterium *Chromatium* can fix small quantities of nitrogen, and

Hamilton & Wilson (71) have been able to show, by using isotopic nitrogen, that *Aerobacter aerogenes*, which has long been suspected of being a nitrogen-fixer, can fix small amounts anaerobically in a well-buffered medium.

Microorganisms and Inorganic Soil Constituents

Evidence continues to grow that microorganisms can make various inorganic nutrients available to plants by bringing them into solution. Bromfield found that several common species of soil bacteria, *Bacillus circulans*, *Bacillus megaterium*, and *Aerobacter aerogenes*, could reduce ferric compounds in the presence of a suitable hydrogen donor and so increase the available iron (and manganese) in soils (22). Aristovskaya points out that the acid produced by some microorganisms may be an important agent of soil formation, especially in podzols. She found that the microflora from podzols was mostly composed of species which grew best in media poor in nutrients, and that several fungus species produce more acid in poor than in rich media (5). Uarova found bacteria in the rhizosphere of wheat plants, which could decompose calcium phosphate, and which increased the water-soluble phosphorus in a compost (219).

Butlin & Postgate have reviewed the sulphur cycle in nature, and have pointed out the economic importance of organisms which produce actual sulphur (28, 29). Quispel, Harmsen, & Otzen report on the oxidation of pyrite in newly-reclaimed marine soils; only the second stage of the process, the oxidation of sulphur to sulphate, is carried out by bacteria, but this reaction stimulates the primary chemical oxidation of the sulphide to sulphur (170). Oxidation of sulphur in Kansas soils has been studied by Moser & Olsen (149).

The Breakdown of Introduced Organic Compounds

There are few substances which are so insoluble, or so toxic, that soil microorganisms cannot dispose of them. As is well known, even such unpromising carbon sources as the straight-chain hydrocarbons can be broken down by bacteria. Ladd (111) describes a *Corynebacterium* which oxidizes such compounds, and Konovaltschikoff-Mazoyer & Senez (109) obtained several hydrocarbon-decomposing pseudomonads from the oil-soaked earth near the Marseilles refineries (111, 109). Levine & Krampitz found a *Corynebacterium* which could oxidize acetone (121).

Arnaudi, Canonica, & Treccani have reviewed the whole subject of the breakdown of hydrocarbons, and also of aromatic compounds (6). Treccani *et al.* (218) and Murphy & Stone (150) studied the breakdown of naphthalene, and Walker & Wiltshire (230) that of chloro and bromo-naphthalene, by soil bacteria. Webley *et al.* found that *Nocardia opaca* breaks down the side chain of phenyl-substituted fatty acids by *beta*-oxidation (237).

Many studies deal with the decomposition of the hormone herbicides and related compounds. Audus (7) has published a series of papers on the breakdown of 2,4-dichloro-phenoxyacetic and 4-chloro-2-methyl-phenoxya-

cetic acids (better known as the weedkillers 2,4-D and MCPA). Jensen & Petersen (99) describe two species that can break down 2,4-D, and Stapp & Spicher isolated a new 2,4-D decomposer, *Flavobacterium peregrinum* (192). Audus & Symonds (8) studied the kinetics of breakdown of 2,4-D by their previously-isolated strain of *Bacterium globiforme*, and Walker & Newman found that the same compound was attacked by a species which they tentatively identify as a *Mycoplana* (231).

Steenson & Walker have isolated, from soil, a *Flavobacterium* that can break down 2,4-D, an *Achromobacter* which attacks both 2,4-D and the related compound MCPA, and another *Achromobacter* which attacks *para*-chloro-phenoxyacetic acid (194). Rogoff & Reid (174) find that a *Corynebacterium* can break down 2,4-D, and Jensen & Gundersen (98) describe another that decomposes aromatic nitro-compounds. Walker has measured the breakdown of chlorophenols in soil by the percolation method (229).

INTERRELATIONS OF THE SOIL POPULATION

Thornton in his Leeuwenhoek lecture (216) has discussed the various possible effects which the different groups of soil microorganisms may have among and between themselves. It is of course clear that the relations between microorganisms in soil may be either mutually beneficial or harmful, but comparatively little attention has been paid to the former aspect. The process of dissimilation of a compound in soil is usually by stages so that an organism that carries out the initial stage may provide nourishment to different groups, but these food chains are usually complex, and their analysis awaits knowledge of the chemical pathways along which the compounds concerned are broken down (79, 194, 230). Some organisms benefit their neighbours by synthesising growth substances such as vitamin B_{12} (27).

Most attention has been paid to the competition between microorganisms in soil and this aspect has received increasing attention lately because of its importance to the control of root pathogens. Most of these are fungi, hence soil microbiologists have been concerned with the isolation and study of soil organisms antagonistic to fungi. These include other fungi, actinomycetes and bacteria. Morton & Stroube found that 3.5 per cent of the fungi, 1.7 per cent of the actinomycetes and 0.2 per cent of the bacteria isolated by them from soil were antagonistic in agar cultures to *Sclerotium rolfsii* (148). Luke & Connell found that 16 per cent of the fungi and 3.6 per cent of the bacteria isolated by them from sugar cane soils were antagonistic to *Pythium* (126). Soil bacteria producing antibiotics are relatively uncommon but are of interest as being possibly easier to use as inoculants for biological control. Chinn described a pseudomonad, found to predominate on a sample of wheat grain, that was strongly antibiotic to *Helminthosporium* and showed considerable antibiotic antagonism to *Fusarium* and to a wide variety of bacteria (36). Antibiotic fungi are more abundant in soil. Jeffreys *et al.* found that of 65 fungal species isolated from sandy soils, about half produced antibiotics, usually active against both bacteria and other fungi. About 45 per

cent of the species that were widespread or locally abundant antagonised other fungi, but only 15 per cent of the rare fungi did so, suggesting some selective advantage in antibiotic production. None of the ten species of Phycomycetes showed such antagonism (92).

On platings of soil suspensions, antagonism of actinomycete colonies towards nearby colonies of fungi is often noticeable so that the former group has attracted particular attention as antagonists to fungi (217). Thus Stessel, Leben & Keitt sprayed platings of soil dilutions with suspensions of the fungi *Glomerula, Colletotrichum, Helminthosporium* and *Verticillum*. Out of some 70,000 colonies developing on the plates, 170 were antagonistic and, of these, 80 per cent were of actinomycetes (196). Fungi vary greatly in susceptibility to actinomycete antibiotics and this specificity is found even amongst closely related strains. Buxton & Richards tested sixteen soil actinomycetes for activity *in vitro* against eight pathogenic strains of *Fusarium oxysporum*. Three of the former were inactive, nine inhibited all the *Fusarium* strains equally, but four of the actinomycetes showed specific differences in degree of inhibition according to strain of *Fusarium*, which they could be used to distinguish (30). One of the actinomycetes that showed specific activity was identified as *Streptomyces albidoflavus*, shown by Skinner also to be strongly antagonistic to *Fusarium culmorum* (188). Antibiotics produced by actinomycetes are also active against some other actinomycetes. Peterson studied the cross antagonisms amongst a collection of 46 actinomycetes, all of which were active against *Streptomyces scabies*. They varied greatly both in the number of the other strains that they would antagonise, and in the number to which each was susceptible. These results show the need for careful selection of antagonistic actinomycetes resistant to the attack of other species, if it is desired to establish them in soil to control a pathogen (161).

The potential usefulness of antibiotic-producing organisms for biological control in soil depends not only on the feasibility of establishing them in fresh soil, but also on their ability to produce antibiotics in effective concentration in field soil and on the activity and persistence of these antibiotics in the soil. Even in sterilised and partially sterilised soil Grossbard (68) found that *Penicillium patulum*, *Aspergillus clavatus* and *Aspergillus terrens* only produced antibiotic in detectable amounts where available carbon sources were added, while Gregory *et al.* found only traces of activity in soil cultures of *P. patulum* (67). Clear evidence for the production of a specific antibiotic in unamended soil was obtained by Gottlieb & Siminoff (65) in the case of chloromycetin and by Wright for gliotoxin (247). But attempts to show this with other antibiotics have generally been negative. Even if an antibiotic is formed in soil a variety of environmental factors may limit its activity or result in its rapid destruction. These factors have been studied in the case of several antibiotics by Gottlieb *et al.* (64) and Hessayon (80), while Jeffreys investigated the behaviour of 10 antibiotics in soil (91). The principal factors causing inactivation appear to be (a) the adsorption of basic antibiotics by the soil, (b) instability at the pH of the soil, (c) chemical reaction with some

soil component and (d) microbial decomposition. Such results have caused the view to be expressed that antibiotic action is unlikely to be important in soil. On the other hand failure to detect antibiotics in soil cultures of organisms known to be capable of their production may be due to a lack of selectivity in the methods used for their detection, most of which have involved extraction from the soil. Stevenson (198, 199) has developed a sensitive method for detecting the production of antibiotics by cultures of actinomycetes in sterilised soil. Agar-coated microscope slides seeded with spores of *Helminthosporium* were buried in the soil culture, which inhibited their germination to varying degrees as compared with a sterile soil control. Evidence that this effect was in fact due to the actinomycete antibiotic was obtained by studying the effect with pregerminated spores. Some of the actinomycetes employed produced quite characteristic types of deformation of the fungal hyphae *in vitro*, and these specific effects were also shown on the hyphae from pregerminated spores buried in soil culture. Moreover the same specific effects were produced by *Streptomyces antibioticus* and by the antibiotic actinomycin, which it produces, both *in vitro* and in soil.

Another important cause of antagonism between microorganisms in soil is competition for some limiting nutrient which may be exerted whether or not a competing organism produces an antibiotic. Skinner studied the growth of *Fusarium culmorum* in the presence of *Streptomyces albidoflavus* which produces an antibiotic very active against this fungus but one that is strongly adsorbed by bentonite. In sand culture, the actinomycete could strongly inhibit the fungus, even preventing the germination of its spores. When bentonite was added to the sand in excess, normal germination of the fungal spores took place but growth of the fungal mycelium was still much reduced by the actinomycete, and this Skinner attributed to nutrient competition. He found that competition increased with the concentration of glucose supplied (188, 189).

Antibiotic organisms may merely arrest growth of a susceptible organism but some, such as the Myxobacteria (Noren) actually lyse and can thus feed on the organisms attacked (153). Such lysis of fungal mycelium by actinomycetes has also been observed by Skinner (188). A dramatic example of direct attack of one organism on another is that of fungi that catch and destroy eelworms. In a survey of these fungi from 49 English soil samples, Duddington found that the most abundant were *Arthrobotrys dactycoides* and an unidentified species that would not form spores (51). Equally remarkable is the amoeboid organism found by Weber, Zwillenberg & van der Laan that attacks and digests nematodes (236).

Evidence is accumulating that fresh soil contains a fungistatic factor, destroyed by heating, that inhibits the germination of spores of a number of fungi. This has been found by Dobbs & Hinson (47), Chinn (35) and Jeffreys & Hemming (93) while extracts of fresh peat have been found to be strongly inhibitory to bacterial growth by Pochon & de Barjac (165).

Stover (203) and Sanford (184) have reported on the survival of differ-

ent plant-pathogenic fungi in soil, and Park (157) has found that alien species of fungi introduced into soil do not withstand competition from the indigenous microflora as well as do species native to the soil. Cuthbert *et al.* report on the survival of bacteria which are indicators of faecal pollution (38). Vilas, Tejerina & Rubio put forward the interesting hypothesis that some bacteria may be present in soil as filterable forms, perhaps less vulnerable to antibiotic attack (222).

When the difficulties of establishing an antibiotic organism in soil, of ensuring conditions therein for adequate antibiotic production, and of choosing an organism whose antibiotic is active and persistent in soil are considered, it is small wonder that attempts at biological control of root disease have so far met with very limited success, though the number of positive results has been by no means negligible. This field has been well reviewed recently by Wood & Tveit (246).

Since the root surroundings are the site at which biological control might most likely be effective, more search for antagonistic organisms in the rhizosphere would seem worth while. This is the more so since several antibiotics are known to be taken up by the roots wherein they remain active and may be protected from the hazards to which they are exposed in the soil (168, 201).

INTERACTIONS OF PLANTS WITH THE MICROFLORA

The Rhizosphere

The micropopulation of the rhizosphere has continued to attract well-deserved attention. The rhizosphere effect can be particularly well studied in soils poor in organic matter, where the population of the control soil is sparse. Sand dunes in course of reclamation, for instance, show a gradual increase in numbers of microorganisms; Milosevic (145) has shown this for dunes on the coast of Yugoslavia, and Webley *et al.* (238) showed that the plants colonizing the partly reclaimed dunes on the Scottish coast had, in their rhizospheres, very much larger numbers of microorganisms than were found in the surrounding sand. It was known from early work that numbers of organisms in soil fall off rapidly with distance from the root; some data on this point have been provided by Glathe *et al.*, who buried Cholodny slides among and near the roots of growing plants (60). The validity of the qualitative differences in bacteria claimed to exist as between rhizosphere and control soil were questioned by Wallace & King as a result of a study of cereal plots (232). This gave rise to a paper by Lochhead & Rouatt (124) who criticised the taking of the control samples by Wallace & King and gave an interesting summary of data from a considerable number of experiments showing qualitative differences between rhizospheres and control soil, in the percentage of bacteria requiring amino acids.

Compounds secreted by the roots of plants must obviously have a very marked influence on the rhizosphere. Samtsevich found the highest numbers of organisms in tree rhizospheres during the autumn, and suggests that root

secretions may be most abundant at this time (183). Kerr found that some roots secrete a substance stimulating the growth of fungi (104). Good progress in the study of the nature of the compounds secreted by roots is reported by Katznelson, Rouatt & Payne (103) and by Rovira (178). The former authors found that drying plants to wilting point and remoistening greatly increased the secretion of amino acids, and enabled that of reducing substances to be detected. They suggested that alternate drying and wetting of soil may produce a similar and possibly important effect in the field (103). The presence of growth factors in the rhizosphere can also be due to their synthesis by microorganisms, as is shown by the work of Lochhead and his colleagues referred to above (27). The specific effects on the microflora of living roots in contrast to dead plant material is shown in a paper by Rouatt & Lochhead, who found higher percentages of bacteria requiring amino acids in the rhizospheres of wheat, oats, flax, timothy, red clover and lucerne, than in the control soil. When materials from the same plants were added to and allowed to decompose in the soil, no important changes in the proportions of different nutritional groups of the soil bacteria could be detected by the same technique, with the interesting exception of lucerne. The addition of lucerne increased the proportion of bacteria requiring B_{12}, known to be produced by *Rhizobium meliloti* in exceptionally large amounts (27, 177).

Rhizobium

The wide and varied field covered by studies of legume nodules and of *Rhizobium* has been the subject of review by Wilson & Burris (245), Thornton (215), Allen & Baldwin (2), Virtanen (225), and Nutman (154), the last dealing more particularly with the relation of host plant physiology and genetics to infection and nodule behaviour. It is not therefore proposed to attempt any cover of this field in the present review. But one aspect of it has come into prominence recently and may be briefly discussed, because of its bearing on the ecology of microorganisms in soil. This is the competition between strains of *Rhizobium* in the relation to the establishment of a culture used for inoculation. The practical importance of this depends on the existence of areas where local strains of *Rhizobium* that are ineffective on the crop that is to be sown, are prevalent in the soil.

Among clover nodule bacteria there is a tendency for strains isolated from subterranean (*Trifolium subterraneum*) crimson (*T. incarnatum*) or cluster (*T. glomerulum*) to be ineffective on white clover *T. repens* and *vice versa* [Baird (10), Vincent (223)]. One cause of the prevalence of strains ineffective on the crop to be sown may thus be the natural prevalence or frequent cultivation of other clovers on which these strains are effective. Thus Vincent found that in the Lismore region of New South Wales, where *T. repens* is the commonest species, nearly all the strains of *Rhizobium* tested were ineffective on *T. subterraneum* and on *T. incarnatum* (223). It may, therefore, be necessary to introduce an effective strain where it is desired to sow a crop variety on land in which the predominant strains are ineffec-

tive, and this must be done in competition with the strains already existing in the soil. It is known that strains of *Rhizobium* differ markedly in the ability to compete with each other for growth and nodule formation in the host. In choosing a strain for use as an inoculant it may be necessary to select one not only effective on the crop but dominant in establishing itself in the crop in competition with the strains already in the soil. The study of strain establishment has been facilitated by extensive surveys that have been made of the antigen relationships of clover nodule bacteria. The use of serologically identifiable strains of *Rhizobium* has enabled the percentages of nodules produced by each of a mixture of strains or by the inoculant strain in field trials, to be determined. Read, using this method in field trials distributed over Great Britain, found that strains used to inoculate *T. pratense* differed greatly in the percentage of the total nodules that each produced in the crop and that the most successful strains in this respect differed with the locality (171). Vincent and his colleagues, using single strains and mixtures of them as inocula, also found that strains differed markedly in the percentages of the nodules that each produced, and also in their effect on crop growth. [see Vincent & Waters (224), Jenkins, Vincent & Waters (94) and Baird (10)].

The factors involved in competition for nodule formation are complex. They involve some, inherent in the host plant and the bacterial strain, that influence the process of infection. These are discussed by Nutman (154). Strains of *Rhizobium* also differ in ability to compete with each other outside the root and are variously affected by other components of the soil micropopulation. These include antagonistic organisms [Hely *et al.*, (75)], but Harris also found organisms in the rhizosphere which increased nodule numbers produced by a strain of clover *Rhizobium* in *in vitro* culture (72). These environmental factors will influence the proportions of different strains in the rhizosphere, and hence the chances of infection of each strain. Plants can receive sufficient effective nodulation by an effective strain even when also bearing ineffective nodules, as was shown by Burton, Allen & Berger with *Phaseolus* (26). On the other hand, Harris, in pot experiments in sterilised soil and sand, found considerable competition between effective and ineffective strains as judged by growth of the plants (73). The relative numbers of effective and ineffective nodules produced by competing strains is probably of importance beyond some limiting ratio, which may differ with the type of nodulation of each strain and under different conditions. It should be remembered that the number of cells of *Rhizobium* introduced by seed inoculation may be small compared with those in the soil, so that, when the latter are ineffective, a competitive inoculant strain may be needed to ensure a sufficient percentage of effective nodules.

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SEROLOGICAL IDENTIFICATION OF PLANT VIRUSES AND SEROLOGICAL DIAGNOSIS OF VIRUS DISEASES OF PLANTS[1,2]

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Introduction

For a proper control of any disease an early, quick, and reliable diagnosis is essential. Virus diseases of plants are no exception to this rule. Special difficulties arising here are: the symptoms shown by the diseased plants often afford only poor directives; in many cases, they cannot be trusted at all and sometimes they are masked or entirely lacking (symptomless carriers); Moreover, the postulates of Koch cannot be applied to viruses, as their cultivation as a pure culture on artificial media is impracticable. Furthermore, the presence of a virus in a plant cannot always be directly demonstrated.

Although electron-microscopy has become a valuable asset for the extension of our knowledge of viruses, and also has been very helpful in serological research [Bawden & Nixon (9); Black *et al.* (24); Rawlins *et al.* (76); Steere & Williams (98); Wetter & Brandes (106); Williams (107)], its application to clinical diagnosis of virus diseases of plants [de Bruyn Ouboter *et al.* (30)] becomes too complicated.

In many cases the serological diagnosis has rendered a satisfactory solution of the problems involved.

Serological Principles

The principles of serology are simple. If an antigen is parenterally introduced into the body of a rabbit, it may stimulate the formation of specific antibodies. The blood serum of the rabbit contains those antibodies which may specifically react in some observable way with the antigen that stimulated their formation. Plant viruses can be used as antigens and, in a suspected plant, their presence can be demonstrated with homologous antisera.

[1] The survey of literature pertaining to this review was completed in January, 1957.

[2] The following abbreviations are used: BV (Bukett virus); IVM (intraveinal mosaic); PAV (pseudo aucuba virus); TMV (tobacco mosaic virus); TRSV (tobacco ringspot virus).

The reaction between antigen and antibody can be performed in different ways (see later). It is generally accepted that it is most probably the virus itself that acts as an antigen [Bawden (7); Chester (35)].

History.—Serology has been developed as a branch of medical science and has played an important part in the study of immunology. See, among others, Bawden (7), Boyd (25), Carpenter (32), Kabat & Mayer (43), Luria (58), Smith (89), and Topley & Wilson (102).

Its application to the study of plant viruses started with the publication of Dvorak (40). He showed that a virus changed the precipitability of the globulins in potatoes. The significance of the serological reactions for the study of plant viruses and plant virus diseases was shown for the first time by the pioneer work of Purdy-Beale (73, 74, 75). Among the early workers in this field are: Bawden (6), Birkeland (21), Gratia (41), Matsumoto & Somazawa (61), and especially Chester (35), who published a great many papers on this subject. In his "Critique of Plant Serology," he gave an excellent survey of the work done by himself and by others on plant serology up to 1937. Since then, serological investigations on plant viruses have been carried out in different centres of plant virus research all over the world.

Although serological diagnosis is not a panacea for all the difficulties encountered by the virologist, yet it has become an indispensable link in the study of plant viruses and the diseases they cause. The immunological studies of the viruses and other methods of virus research have had a mutual complementary effect. Serological methods have helped to elucidate the knowledge of the viruses, but their scopes are inseparably linked up with the progress in the knowledge of the viruses, and of the biochemistry of the proteins of healthy, as well as of virus-diseased, plants. Every progress made by the biochemist in the purification and concentration of plant viruses has been, and will be, a godsend to the plant virus serologist in his struggle with the problems encountered. Team work of biologists, virologists, and biochemists is very essential, indeed.

Merits and possibilities.—The main merit of the serological reaction is that it is specific and objective and, apart from this, it is independent of the symptoms shown by the diseased plants. It enables research workers to study the spread of a virus inside a plant after its infection [Matsumoto & Somazawa (61)], to acquire a quantitative measure of the amounts of the virus in sap of infected plants and in purified suspensions [Beale & Lojkin (14); Chester (33); Purdy-Beale (75); Steere (96)], to study the concentration of the virus in different stages of development of the plants [Bartels (4, 5); Bercks (18); Bercks & Querfurth (20)], and to identify and to study relationships between viruses [Chester (34); Purdy-Beale (74), and many others]. The antisera can be stored and kept available for the testing of suspected plants at any time of the year. Compared with the classical infection experiment it saves the time lost by incubation; e.g., in the case of flowerbulbs this requires almost a whole year, because no current season symptoms can be

anticipated. In horticultural and agricultural crops, its quickness enables an early elimination of infected plants. Especially with vegetatively propagated stocks as potatoes and flowerbulbs, the parent plants to be used for propagation can be tested within a short period before the time of lifting. Thus it has become a useful method of roguing seed-potato stocks. Timian *et al.* (101) applied serological tests to eliminate symptomless carriers in their breeding program, with the aim of selecting plants immune to virus X after a mass inoculation of seedlings.

By the application of serological research unknown disease-causing viruses have been detected, e.g., the virus S of potatoes, another unknown potato virus in Holland, unknown viruses of Bagnall & Bradley (1), Köhler (51, 52), and recently it was very helpful to Bagnall *et al.* (2) in discovering the M virus in IVM Irish Cobbler. Although their research was not exclusively focused on the serological aspect, they state: "Nevertheless it was obvious that serology was one of the most useful and reliable tests employed in these studies." The neutralizing effect of antibodies on their homologous antigens are used for the identification of viruses. The possible effect of heterologous antisera or normal sera on the antigen, and the influence of these sera on the reaction between virus and host plant, have to be carefully considered [Bawden (7); Chester (33); Kassanis (44)].

Some difficulties and limitations.—Active antisera cannot be prepared to all viruses. Bartels (3) and Beemster (15), among other authors, did not succeed in preparing an antiserum to the virus of leafroll in potatoes. Claims of having prepared such an antiserum must be ascribed to the presence of another virus, probably mainly virus X or virus S of potatoes, of which the investigator was not aware. What can be the reason for this failure? The statement that the virus is not antigenic does not explain anything. Possibly, the concentration of the virus is too weak, or it is lost because of its instability when the parts of the plant that should contain the virus are crushed or extracted. It has been presumed that the body temperature of the immunized rabbit may be too high and that coldblooded animals should be used [Chester (35); Bartels (see reference No. 15 for discussion article of Beemster)]. Kassanis (45) failed to prepare an effective antiserum to dandelion yellow mosaic virus and to other viruses causing disease in lettuce. Many others, the authors among them, have failed in many instances to get an effective antiserum, but as a rule, negative results are not published. Chester (35) established that there is a correlation between mechanical transmissibility and antigenicity, implying that viruses transmissible only by insects are not antigenic. In general, this may be true, yet Kleczkowski & Watson (49) and van Slogteren *et al.* (88) could prepare a specific antiserum to the virus of sugarbeets which is very difficult to transmit mechanically [Costa & Bennet (38); Kassanis (46)]. The authors prepared a specific antiserum to the virus causing a mosaic disease in hyacinths (87), although up to now it could not be transmitted mechanically. Since then, Black *et al.* (23, 108) have prepared

antisera to viruses transmitted by leafhoppers. Other cell constituents may more particularly promote denaturation of virus proteins when the plants are crushed for extraction of the virus. No antisera could be prepared to viruses of strawberries and other plants known to be rich in tannins. Cornuet *et al.* (37) applied reducing agents and oxidase inhibiting substances in purification experiments on dahlia mosaic virus. In studying extracts from milled fibre incubated with trypsin of tomato plants infected with Bushy stunt virus, Bawden & Pirie (12) found that chromoprotein, inhibiting precipitation with antisera, can be coagulated by freezing and may thus be removed. Alternatively, Burger (31) found that in the purification of Yellow streak mosaic virus of wheat, the loss of stabilizing constituents may lead to inactivation, the addition of protecting substances such as gelatin to partially purified virus suspensions preserving activity. Thus the possibilities of purifying and concentrating the virus antigens before specific antisera are prepared, are of great importance. Hence, the work of Bawden & Pirie (11), Black (22), Brakke (27, 28), Price & Black (71, 72), Stanley (92), Steere (97), Wyckoff (109), and many other workers in this field is of high value to plant-serological research.

Preparation of antisera.—Fewest difficulties in the production of antisera are encountered if injection can be carried out with relatively thoroughly purified and concentrated virus suspensions as, e.g., those which can be prepared from tobacco mosaic virus, Southern bean mosaic virus, or potato X. But not all plant doctors can restrict their work to viruses that can be fairly thoroughly purified and concentrated. However, it is evident from the results with many of the less stable viruses as, e.g., sugarbeet yellows, that active antisera can be prepared by injecting relatively crude suspensions made from extracts of infected plants. The resulting antisera contain a higher proportion of antibodies to normal plant proteins which consequently have to be removed by absorption of the sera with an excess of sap from virus-free plants (Principle of Castellani). An alternative way to evade most of these difficulties is by the propagation of the virus antigen in another host plant, not serologically related to the plant to be tested for the presence of the virus. Reversing this procedure, Dounin & Popova (39) prepared an antiserum to the juice of a healthy plant and absorbed this antiserum with the juice of virus-infected plants. In this way, the juice of the diseased plant was freed of normal plant antigens, and was subsequently used to immunize rabbits. Kozlowska (54) applied the same principle. Van der Veken (104) precipitated the fraction containing virus-specific antibodies from antiserum-sap-mixtures by addition of alcohol at low temperatures. Antibodies isolated in this way can be preserved by freeze-drying. In all cases it is essential that the substances toxic to animals as, e.g., alkaloids, be eliminated by dialysis of plant extracts. If toxicity is doubtful, the authors test their preparations on mice, injecting small doses in the tail veins, before a bigger animal is used. Toxic substances in extracts of freesias and carnations are traced in this way (53, 80).

The intravenous mode of injection is applied by most workers, but others are also used. Bagnall *et al.*, working with potato viruses X, S, and M, injected intraperitoneally (2). Comparing (unpublished) results of intraveous and subcutaneous injections of potato virus X into horses, the authors found with the latter method that antisera were obtained showing rather high precipitin dilution endpoint titres when mixed with clarified sap from infected potato or tobacco leaves, but these antisera were lacking in "avidity" when they were mixed with increasing dilutions of the antigen, in comparison with the antisera prepared by intravenous injections. The occurrence of a high percentage of so-called incomplete or blocking antibodies may explain these differences.

In recent years the production of antisera to some of the less stable plant viruses has been successful either by the use of new methods of purification of antigens or by applying the adjuvant technique of immunization, or by a combination of both methods. Moorhead (63), working with Barley stripe mosaic and Brome mosaic viruses, administered a "booster" dose of virus in an adjuvant (mineral oil) after initial injections of virus in buffered saline, as well as a single injection of the virus in an adjuvant. Her results suggest that the maximum antibody response following upon the injection in an adjuvant remains high for a longer time than after saline injections, though that response is attained after a longer period. Black & Brakke (23) purified the leafhopper-transmitted wound-tumor virus by zonal centrifugation and zonal electrophoresis in sucrose solution density-gradient columns [Brakke (27, 28)], the purified virus being emulsified with mixed Bayol F mineral oil and Arlacel A as adjuvant. Antisera produced in rabbits by injection into the large muscles of the hind legs showed precipitin endpoints up to 1:1600. By applying the same techniques of purification and immunization, Wolcyrz & Black (108), obained antisera to several strains of potato yellow dwarf virus, with dilution endpoints as high as 1:1280 against homologous strains, lower titres with heterologous strains being indicative of minor antigenic differences. Purification methods based on the solution of viruses in the aqueous phase of a chloroform-water emulsion as described by Schneider (79) should also be taken account of. Thus, the authors (83) attained a partial purification of potato virus S by centrifugation of extracts from infected potato leaves mixed with chloroform, the virus being found in the aqueous phase after centrifugation, the chlorophyll being dissolved in the chloroform layer. Recent (unpublished) results indicate that chlorophyll and other constituents can also be removed by percolating chloroform or other organic solvents (alcohol, acetone, and ether) through virus-infected leaves after the latter have been freeze dried and disrupted. Steere (97) purified tobacco ringspot virus by adding two volumes of a 1:1 mixture of *n*-butanol and chloroform to one volume of crude juice from infected plants. He stirred the mixture for 15 min. and centrifuged it for 30 min. before separating the virus from the aqueous phase by alternate low and high speed centrifugation.

In most cases rabbits have been used for preparing antisera to plant

viruses. Among other animals used for this purpose may be mentioned: sheep and pigs, Stapp (93); chickens, Newton & Edwards (68); frogs, Bartels (15); and horses, van Slogteren (86, 87). Immunization of horses enabled the authors to procure enough antisera to the potato viruses X and S for testing annually about 1 million potato plants in stocks intended for the production of virus-free seed potatoes. Antisera to potato virus X were prepared by injecting an eleven-year-old gelding intravenously (vena jugularis) with purified virus suspensions. A first series of 19 injections, doses of 300 ml. being given twice a week, yielded 10 litres of antiserum with a precipitin dilution end point titre of 1:640 when tested against clarified sap of infected potato plants. Three years later a second series of injections into the same horse yielded 47 litres of antiserum with titres up to 1:1280. Antisera to potato virus S were prepared in a three-year-old mare, suspensions purified from sap of infected potato leaves being injected intravenously. A first series of 15 injections in increasing doses from 200 to 300 ml. yielded 25 litres of antiserum with precipitin dilution end point titres varying between 1:320 and 1:640. A second and a third series of injections into the same horse yielded respectively 35 and 14 litres of antiserum with titres varying between 1:640 and 1:1280. It was noticed that after the third series of injections the titres of serum samples remained on a high level for a longer time than in the case of samples taken after the first and second series.

Serological Methods

Within the space allotted to this survey it is not possible to deal with all the aspects of serological methods. For assay methods we may refer to the excellent survey by Steere (96), who includes serological assay methods in the biological and biophysical approaches to this particular field of research.

Above all it should be realised that however sensitive the serological test may be, it cannot compete in sensitivity with the infection test. For the highly sensitive but delicate and complicated method of complement-fixation we may refer to Kozlowska (54), Moorhead (63), Tall *et al.* (100), Weaver & Price (105), and others. Indeed, according to Chester (35) the complement-fixation test may be the most delicate and specific of all immunological reactions employed in plant serology, its complications rendering it often less useful again to the plant doctor who cannot restrict his research to viruses, as highly purified as tobacco mosaic virus. If other complement fixing constituents are present in fluctuating concentrations, the results will vary too much, and this may be the reason that some investigators claim that their slide-flocculation tests are even more sensitive [Limasset (56); Murayama *et al.* (67)].

Most plant virologists apply the precipitin method, in many different ways modified to their convenience, a special technique being developed for a particular plant-virus combination that is to be analysed. Every worker develops a preference to and an exceptional skill in the technique to

which he is accustomed, notably so if he developed it himself. Most widely applied is the preciptin reaction in tubes of 7 mm. diameter, 1 ml. of diluted antiserum being mixed with 1 ml. of the centrifuged sap of plants to be tested (at various dilutions), whereafter the tubes are immersed in a water-bath at about 37°C. Zaitlin *et al.* (110) working with Cattleya orchids, employ a ring test, layering a small quantity of previously clarified plant extract over an equal quantity of diluted antiserum in four mm. glass tubes.

Microprecipitin techniques have been developed in order to economize on sera and to simplify the manipulations. They have been applied in several modifications by Dounin & Popova (39), Jermoljev & Hruska (42), Matsumoto & Hirane (60), Roland (77), van Slogteren (88) and Stapp (93). The junior author (81) working with flower bulbs, sugarbeet-yellows and potato viruses, mixes droplets of diluted antiserum with equally sized droplets of clarified plant sap on the bottom of a Formvar coated petri dish, thus preventing spread of the mixed droplets. By pouring paraffin oil over them, evaporation is impeded, and in most cases incubation is carried out at 37°C. From 60 to 100 of such mixed droplets can be put in a single petri dish. Samples as small as 0.005 mm.[3] can be tested in this way as has been shown by van Soest & de Meester-Manger Cats (91), who tested stylet droplets of the aphis *Myzus persicae* on tobacco mosaic virus, (TMV). No positive reactions took place when antiserum to TMV was mixed with stylet droplets of aphids feeding on TMV-infected plants. Yet the reactions of equally sized droplets of sap from such plants were positive, indicating that no virus can enter the stylets. The salivary sheath may be a barrier. Stapp *et al.* (95) working mainly with potato viruses, employ paper for absorbing measured amounts of antiserum before being dried. Small round pieces ("leaflets") are punched out which can be stored until needed. For tests, a leaflet is stirred with a few drops of saline, and the centrifuged sap of a plant to be tested is added and mixed. Microdrop reactions described so far are usually read under the microscope with darkfield illumination, positive reactions being identified as white flocculations standing out against the dark background.

The junior author (82, 84) applied the gel-diffusion technique of Ouchterlony (69) to the serological analysis of some plant viruses. Plant extracts or purified virus suspensions and corresponding antisera are separately pipetted into basins or small holes present in thin layers of agar, at some distance from each other. Specific lines of precipitate are formed somewhere between these diffusion centres, standing out clearly inside the transparent gel. Patterns of multiple lines may result, representing either several antigenic components associated with infection of one virus as in the case of tobacco mosaic virus, or antigenic complexity if a plant is infected by more than one virus (Fig. 1). In applying the same method Kleczkowski (48) suggests that all components specific to plants infected by tobacco mosaic virus may not be serologically related, as was hitherto assumed. He also demonstrated two serologically unrelated antigens in a crystallized preparation

of tomato bushy stunt virus, not detected in healthy plants. The authors think it is an advantage of the gel-diffusion method that it demonstrates antigenic components separately, which by other methods of approach may get lost during the purification processes or may be overlooked.

Chester was the first to describe the phenomenon of formation of a dense aggregate of chloroplasts and cell fragments when 2 ml. of crude sap, pressed from TMV-infected tobacco leaves, are mixed in a Wassermann tube with the corresponding homologous antiserum. As this simple agglutination method needs no complicated equipment for its application, it has been modified for large-scale testing of plant material by several workers, mainly for selection of virus-free potato stocks. The senior author *et al.* (84, 88) developed a modified technique employing five drops of diluted antiserum mixed on a glass slide with one or two drops of sap of the plant to be tested. In Holland this test is used on a wide scale today for screening the potato viruses X and S in seed potato stocks. Bradley (26) and Munro (65) describe a method based on the same principle for testing potato plants in the field for the presence of virus X. Instead of glass slides Swiezynski (99) used porcelain spot plates with 12 depressions, three drops of the sap to be tested being mixed with antiserum in each of these. The paraffin oil method (81) already mentioned can be applied as a microagglutination test, by mixing drops of antiserum with smaller droplets of the crude sap to be tested. Stapp & Bercks (94) applied sheep erythrocytes as agglutinating substance instead of chloroplasts, for the detection of virus X in extracts of potato tubers. Moorhead & Price (64) developed a sensitive serological test for TMV, using red blood cells of the sheep as indicator of antigen-antibody combination. They added 0.2 ml. of a 1 per cent suspension of cells to mixtures of diluted antiserum and diluted antigen in wells of Lucite spot plates. Positive reactions were identified from a characteristic pattern of settled cells after incubation at 5°C. for 3 hr., the cells being entangled in the antigen-antibody network.

All methods of serological testing are complicated by the occurrence of spontaneous reactions caused by the presence of nonspecific precipitates, or by the spontaneous aggregation of chloroplasts for reasons other than antigen-antibody combination. Mainly these difficulties occur unexpectedly due to the fact that the conditions of the test cannot be sufficiently standardized, living plant materials of different ages and grown under different conditions being involved. In the case of testing purified or partially purified suspensions, salts or other substances added during extraction may also have an unpredictable effect on the tests. For these reasons rigid checking of all tests is indispensable, control reactions with normal sera and with saline being essential. If absorbed antisera are used, normal sera and saline should be treated alike with the same amounts of sap from virus-free plants as are used for the absorption of the antisera. Nonspecific reactions recognized by flocculations with normal sera or saline in the case of precipitin reactions with centrifuged sap may be averted by various treatments such as freezing

and thawing of the sap, by incubation for a few hours at room-temperature, or by adding Na_2HPO_4 to the sap [Bawden & Pirie (12)]. The authors add buffer solutions during extraction of freesias (53).

FIG. 1. Gel-diffusion method. Lines of precipitation between sap from mosaic-infected tobacco plants in basins 2, 3, and 4 and antiserum in the center. Sap from virus-free plants is present in basin 1.

DIFFERENTIATION OF SEROLOGICALLY UNRELATED VIRUSES, AND OF SEROLOGICALLY RELATED STRAINS

It frequently happens that crops are infested by two or more viruses. Especially in those cases where the presence of each single virus cannot be separately ascertained on the basis of symptoms only, serological methods have been helpful in distentangling these complexes. Kassanis (47) isolated four viruses from carnation plants, three of which could be differentiated serologically. All three were serologically unrelated, as could be corroborated by other research methods. A serological relationship exists between his carnation latent virus and a virus occurring in many potato varieties, which since has been identified as a strain of virus S. Van Koot *et al.* (53), working with virus-diseased freesias, identified *Phaseolus* virus 2 as a cause of disease by means of antisera prepared with extracts from infected broad beans. When antisera were prepared with extracts from infected freesias, it was revealed, however, that in many cases a second unrelated virus played a role, either alone or in combination with *Phaseolus* virus 2. Potato virus S was originally

detected [de Bruyn Ouboter (29)] when an attempt was made to prepare antisera to potato virus A, by injecting rabbits with extracts from A-infected potato leaves. The resulting antisera precipitated strongly with the sap of many potato varieties, but not with some known to carry virus A. According to Bagnall *et al.* (2), Bagnall & Bradley (1) prepared an antiserum against the same virus with sap extracts from potato seedling No. 41956. In the paper just referred to (2) the identification, partly by serological methods, of three viruses in IVM Irish Cobbler is described, the potato plants showing interveinal mosaic symptoms caused by virus X, virus S, and a third "IVM factor," which they designated as potato virus M. The latter virus could be isolated by repeated passage through the host *Datura metel.* Cross-absorption tests between heterologous sera pointed to a slight serological relationship between virus M and virus S. In cooperation with A. Roozendaal, the authors prepared an antiserum to a new virus inciting a mild mottle in the variety *Bintje* (results not yet published). From data obtained so far it might be concluded that this new virus is identical to virus M in Irish Cobbler, as there also seems to exist a slight serological relationship between this virus and virus S, but this cannot yet be taken for certain. Viruses causing tobacco necrosis belong to a serologically distinct group, as demonstrated by Bawden & Pirie (10) and by Bawden & van der Want (13).

By applying the complement-fixation technique, Moorhead (63) compare Barley Stripe mosaic virus and Brome mosaic virus isolates (both cereal viruses). As no cross-reactions were observed, she designates these cereal viruses as distinct serological entities. Likewise Tall, Price & Wertman (100) differentiated Tobacco ringspot virus and Tomato ringspot virus as belonging to unrelated groups by the biological cross-protection test and the serological complement-fixation test. Bercks & Gehring (19), using the precipitin technique, determined a close serological relationship between Kohler's (52) ringspot viruses, Bukett virus (BV) and Pseudo Aucuba virus (PAV), differentiating these two from the typical Tobacco ringspot virus (TRSV). Antigenic determinants specific to each strain separately together with a major antigenic fraction common to both viruses could be found by cross-protection experiments. They could only establish a slight serological relationship between TRSV and PAV.

In experiments with virus-infected narcissus plants, (not yet published), the authors serologically disentangled a complex of two viruses, one being the so called "grey" virus inciting a characteristic yellow mottle and hypertrophy of the leaf epidermis, the second being designated as "mosaic," frequently causing only a very mild mottling, and in other cases being masked altogether. As the latter virus occurs together with the "grey" virus as a rule, it complicates the preparation of antisera specific to "grey," because it behaves as the stronger antigen of the two. For the preparation of antisera to this virus it is therefore necessary to start with a careful selection of plants harbouring the grey virus singly.

Zaitlin *et al.* (110), working with virus diseases of Cattleya orchids, concluded that their antigen-antibody system was complex on account of the fact that both antibody and antigen were detected in the supernatant of one of the precipitin reaction tubes during the quantitative precipitin analysis. This implies that more than one antibody was present in the antiserum. These facts corroborated observations made by electrophoresis of virus preparations which revealed two migrating components, and electron microscopic observations, which revealed the presence of two morphological types of rods, a proof of the existence of two distinct viruses in orchids. Relationships among four viruses affecting the genus *Brassica* were serologically demonstrated by Larson *et al.* (55), who differentiated the Turnip virus 1-group from three other viruses affecting the same genus. Attempts to prepare antisera to the latter three were unsuccessful. Cross-absorption experiments and also a comparison of precipitin end point titres between antisera and homologous and heterologous antigens, sometimes point to serological differences between the strains of a virus, although the use of the term "strain" is subject to controversial opinions [Bawden & Kassanis (8), Knight (50)] notably due to discrepancies between the method applied to demonstrate serological relationship and the method used to demonstrate cross-protection or mutual interference of viruses. Matthews (62) studied the two phenomena of cross-protection and serological relationship with strains of potato virus X. Strains indistinguishable according to cross-absorption experiments showed complete cross-protection, whereas protection was incomplete with strains showing serological differences. Bawden & Kassanis (8) demonstrated that Tobacco veinal necrosis virus, although showing a distinct serological relationship to the potato viruses Y and C, does not protect tobacco or potato plants from infection by either of these types. In their opinion the existence of common antigenic groups is of greater taxonomic significance to the determination of relationships than failure in cross-protection. Knight (50) discussed the relationships between the cucumber viruses 3 and 4 on the one hand, and tobacco mosaic virus on the other. Although both groups share some antigenic and morphological properties, he contends that cucumber viruses 3 and 4 should not be looked upon as strains of tobacco mosaic virus, as great differences in other properties are evident, including failure of cross-protection, but more especially related to chemical structure.

Bercks (17) experimented on the protection afforded by a strain of potato virus X inoculated on Samsun tobacco against another superinoculated strain. By a cross-absorption technique each strain could be identified separately, even when both occurred simultaneously in the same plant. His tests indicate that the superinoculated strain gained access to the tissue without inciting any additional symptoms, thus showing that apparent protection against reinfection by related strains need not really exist. Weaver & Price (105) differentiated strains of tobacco mosaic virus by the complement-fixa-

tion method. Moorhead & Price (64), using sheep red blood cells as an indicator of antigen-antibody combination, demonstrated serological differances among the Type and Rosette Strains of TMV and seven mutant derivatives of the Type strain. Perez & Adsuar (70) confirmed a suspected antigenic relationship between potato virus Y and Puerto Rico Pepper mosaic virus by precipitin tests, while cross-absorption tests revealed a minor antigenic component inherent in the Pepper mosaic but not in the Type strain of potato virus Y. Beemster & van der Want (16) established serological relationships between *Phaseolus* virus 1 and 2 by cross-reactions with the homologous and heterologous antisera.

CONCLUSION

Though perhaps too concise and consequently incomplete, this survey may show the importance of serological research to the increase of knowledge of the viruses in their various aspects. Yet the application of this serological information to the diagnosis of plant virus diseases still lags far behind, when one considers the scope of possibilities. [van Slogteren (85, 86, 87)]. In 1949 Bawden (7) stated that up to then serological techniques were being applied to some 15 different viruses. However, the preparation of an antiserum to a plant virus which is useful by its specificity for fundamental research does not automatically imply that it will be adopted for clinical diagnosis of the viruses which may be met in all infected hosts. Frequently virus production can be increased considerably by transmission to a given suitable host, while the virus concentration in the hosts to be tested is too low for a positive serological reaction. Bartels (4) prepared antisera to potato virus A by immunizing rabbits with purified preparations originating from infected tobacco leaves, picked at the stage of highest virus content. This antiserum proved to be of value in identifying the virus in extracts from tobacco, but in infected potatoes the virus concentration was too low for a reliable serological diagnosis. The authors had the same experience with tobacco rattle virus (*Nicotiana* virus 5, Böning) with antisera which were active to extracts from infected tobacco plants and from heavily infected tulips. However, they rendered unsatisfactory results with other hosts carrying the same virus, e.g., stem-mottled potatoes [Rozendaal & van der Want (78)].

Yet the number of effective antisera suitable for the diagnosis of viruses in plants has rapidly increased. Van Slogteren (87) published a list of 25 different antisera prepared in his laboratory, Limasset (57) mentions 23 antisera, 13 of which are not inserted in the list first mentioned, and at least another 15 antisera are mentioned in still more recent literature. In cooperation with Nederlandse Algemene Keuringsdienst, seed potato growers, the authors have secured large propagating stocks of many varieties free from viruses through the application of serological diagnosis. By preparing bivalent or trivalent antisera, the parent plants can be examined for two or

three viruses in one test. At one station a daily capacity of 21,000 tests has been attained by the introduction of an assembly line. About 6,000,000 plants have been serologically tested since this work was taken in hand. Virus X of potatoes is so widely spread in the U.S.A. that it is known there as "the healthy potato virus." Potato virus S is probably just as widely spread all over the world and might appropriately be called "healthy potato virus No. 2." And what to say about the M virus recently detected by Larson *et al.?* The great economical importance of keeping these diseases under proper control will be evident from the fact that the annual loss caused by each of those viruses separately is estimated at about 15 per cent of the crop [MacArthur (59); Munster & Pelet (66); Smith & Markham (90)]. In combination with other mild viruses, the loss can be much higher.

An international exchange of samples of antisera will not only be useful to purposes of identification, classification, and nomenclature of different viruses described in various countries, but also to the identification of different virus diseases of plants. Coons (36) has been able to show with antisera from the authors that the virus yellows of sugar beets was widely spread in some parts of the U.S.A. Likewise Vaughan (103) applied antisera to Virus S in Oregon and, furthermore, samples of our antiserum to virus S was despatched to 24 investigators in 16 countries. Without any doubt the opportunities opened by serological diagnosis of more plant virus diseases will lead to more healthy crops and will give due satisfaction to the scientists who laid the foundation of plant serological research about thirty years ago.

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MICROBIAL DISEASES OF INSECTS[1]

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There are a number of reasons why microbiologists generally may find interest in the microbial diseases of insects. This area of the field of insect pathology has contributed heavily and directly, not only to microbiology itself, but to the fields of agriculture, medicine, and biology as well. Moreover, from an historical viewpoint, it has significantly influenced the development and progress of microbiology. This fact has been highlighted in a recent treatment (116) of the early (up to 1900) history of insect pathology and its applied phase, microbial control. For example, the first experimental proof that disease in any animal could be caused by a microorganism was Agostino Bassi's demonstration that muscardine of the silkworm is caused by the fungus *Beauveria bassiana* (Bals.). This achievement was followed some years later by the illustrious work of Pasteur on the silkworm diseases pébrine and flacherie; Pasteur's introduction to the phenomenon of microbial diseases of animals.

Recent years have seen rapid development of the field of insect pathology, and our knowledge of microbial diseases of insects is fast accumulating. Moreover, the use of microorganisms in the control of insect pests appears to be entering a renaissance. Within the page limitations of this review it is impossible to treat adequately all of the many worthy papers that have recently appeared on the subjects concerned. Indeed, it was found necessary to exclude more than are included. Accordingly, it will be the author's purpose merely to review briefly, and in some respects cursorily, the literature pertaining to a few of the developments in insect pathology and microbial control that appear to be of outstanding significance and importance, or the literature which, for certain subjects, is particularly scattered. Moreover, only those advances that have occurred since the appearance, in 1949, of the last major comprehensive treatment of the subject (108) will be considered. With minor exceptions we shall arbitrarily not attempt to cover the literature on the diseases of the honey bee, most of the specialized literature on the diseases of the silkworm, and certain aspects of virus diseases.

Virus Diseases

Insect viruses and the diseases caused by them have been the subjects of several authoritative reviews in recent years. Indeed, the literature in this field has been so recently and so thoroughly reviewed by Bergold (13, 14), as well as others [*e.g.* Smith (107a)], that to attempt here even a summary

[1] The survey of the literature pertaining to this review was concluded in December, 1956.

of recent developments would be unnecessarily redundant. Since these reviews are readily available, the interested reader is urged to consult them in order to complete the picture of recent developments in insect pathology to be presented here.

Bacterial Diseases

One of the recent and currently active areas of research, as it pertains to the bacterial diseases of insects, is that concerned with the bacterium *Bacillus thuringiensis* Berliner, and its close relatives *Bacillus sotto* Ishiwata and *Bacillus cereus* var. *alesti* Toumanoff and Vago. Especially noteworthy have been the observations by Hannay (58) and others (2, 3, 5, 60, 118) on the protein crystals formed by these organisms at the time of spore formation. It appears that this material is highly toxic for certain insects, especially certain Lepidoptera in the alkaline gut juice of which the toxic principle is soluble, and is responsible for the marked pathogenicity of these bacteria for these insects. The crystals are characteristically diamond shaped (tetragonal), but in some strains the shape may be rhombohedral to cuboidal. Sometimes two inclusions are seen within a sporangium, but this is a rare occurrence; usually each sporangium contains but one crystal. The crystalline inclusions are freed from the cells along with the spores and appear to persist indefinitely.

Hannay & Fitz-James (60) prepared suspensions of the crystals of *B. thuringiensis* free from other material. The protein nature of the crystals was indicated by the fact that such suspensions contained a substance precipitable with trichloracetic acid and which possessed the ultraviolet absorption characteristics of protein, over 17 per cent nitrogen, and at least 17 amino acids, but no phosphorus. For other characteristics of the crystals, as well as the parasporal inclusions of certain other bacteria, the reader is referred to a review of the subject by Hannay (59).

To explain the pathogenic effect of *B. sotto* toxin on silkworm larvae, Angus & Heimpel (7) have advanced the hypothesis that the increase in blood pH that follows the ingestion of the toxin is the immediate cause of the resulting paralysis. As the blood becomes progressively more alkaline, there is an accompanying decrease in the pH of the midgut contents. This suggests a possible "leakage" of alkaline ions from the gut to the blood as a result of poisoning by the *B. sotto* toxin.

Beginning in 1951, Toumanoff & Vago (141) and Toumanoff alone (137, 138) published a series of papers on a spore-forming bacterium responsible for enzootic flacherie of silkworms in the Cévenole region of France. They considered the organism to be a variety of *Bacillus cereus* Fr. & Fr. and named it *B. cereus* var. *alesti*. Toumanoff expresses the belief that it is the same bacillus observed, but not cultivated by Pasteur some 80 years previously, and known in some quarters as *Bacillus bombycis*. The relation of the *alesti* strain to *B. thuringiensis* is indicated by the fact that it bears a crystal in its sporangium. It is differentiated by the fact that unlike *B. thur-*

ingiensis, the *alesti* strain usually produces a rose to purplish-red coloration in egg yolk, and ordinarily is highly pathogenic for the silkworm. While *B. sotto* is also highly pathogenic for silkworms, it does not produce the red coloration in egg yolk. Whether such variable properties warrant distinguishing these three organisms as varieties (*i.e.*, var. *thuringiensis*, var. *sotto*, var. *alesti*) of *B. cereus*, as suggested by Toumanoff (138, 140), or whether the three, as well as the co-called "Anduze" strain, should be classified as strains of *B. thuringiensis*, as maintained by Delaporte & Béguin (25), appears to be debatable. Angus (4, 6) concluded that *B. sotto* and *B. thuringiensis* can be differentiated by means of certain cultural and morphological differences, by a quantitative difference in pathogenicity for the silkworm (*B. sotto* being the more pathogenic), and by a difference in the solubility of their toxins (that of *B. sotto* being soluble at a lower pH). At present there are cogent pragmatic reasons why the designation *B. thuringiensis* should be used to distinguish this crystal-bearing bacillus from *B. cereus* (118). Nevertheless, the matter admittedly becomes complicated from both a taxonomic and physiological viewpoint in the light of Toumanoff's (137) 1956 reported success in producing a crystal-bearing strain from a strain of ordinary *B. cereus*. He claims to have accomplished this by passing *B. cereus* six times through the blood of larvae of the wax moth, *Galleria mellonella* (Linn.).

While most strains of *B. cereus*, as they occur in nature, are not pathogenic for insects (109), some appear to be. In these cases, instead of a crystalline toxin being responsible for their pathogenicity, it appears that their virulence depends upon their vigorous production of lecithinase (produced to some extent by most strains of *B. cereus*) and the pH of the gut of the host insect. Heimpel (64, 65) found that the reaction of the midgut (pH 7.61) and blood (pH 6.49) of the larch sawfly, *Pristiphora erichsonii* (Htg.), was within the pH range of lecithinase activity, thus partially explaining why this insect is susceptible to the particular strain of *B. cereus* concerned, and why this bacterium is not very pathogenic for the forest tent caterpillar with a midgut reaction of around pH 9.64. Larvae of the codling moth, *Carpocapsa pomonella* (Linn.), have also been found susceptible to *B. cereus* (121).

Advances in our knowledge of American foul brood and European foul brood of the honey bee have been concerned primarily with the use of sulfa drugs and antibiotics in the control of these diseases. A number of papers on this subject have been published during the period covered by this review [*e.g.* (31, 53, 69, 70, 71)]. [Papers pertaining to other subjects, but having to do with antibiotics and insects have also appeared in recent literature (91, 94, 114, 117)]. However, since the subject of bee diseases generally is of such a scope as to merit a review by itself, no attempt will be made to include it in the present discussion.

Tashiro & White (129) have shown that larvae of the European chafer, *Amphimallon majalis* (Razoum.), is susceptible to the milky disease organisms *Bacillus popilliae* Dutky and *Bacillus lentimorbus* Dutky. The virulence of *B. popilliae* for the chafer is about the same order of magnitude as it

is for its well known host, the Japanese beetle. *Bacillus fibourgensis* Wille, a bacterium similar to *B. popilliae* and causing "milky disease" symptoms in the cockchafer *Melolontha melolontha* Linn., has been recently described. An unidentified spore-forming bacillus was observed by Bucher (20, 167) to be pathogenic for tent caterpillar larvae. *Malacosoma pluviale* (Dyar) and *Malacosoma americanum* (Fabr.) were readily susceptible, but *Malacosoma disstria* Hüb. was resistant to the bacillus. Other unidentified spore-formers have been found pathogenic for larvae of the cockchafer *Melolontha melolontha* Linn. (= *M. vulgaris* Fabr.) (72, 165, 166) and the oriental cockroach *Blatta orientalis* Linn. (66) in Europe. *Bacillus sphaericus* var. *fusiformis* Gotth. has been reported (89) as the possible cause of a disease in *Bupalus piniarius* Linn. A strain of *Aerobacter aerogenes* (Kruse) was found by Stevenson (122) to be responsible for a higher percentage of mortality in laboratory stocks of the desert locust, *Schistocerca gregaria* Forsk. Pesson, Toumanoff, & Hararas (95) made a bacteriological study of three new species of coliform bacteria they found responsible for outbreaks of disease in three species of xylophagous insects which were being reared in the laboratory. An interesting infection of the midgut cells of *Solenobia triquetrella* F. R. caused by an unidentified gram-negative small rod has been described by Puchta & Wille (97a). *Bacillus dendrolimus* Talalaev is the name given to a newly described sporeforming pathogen of the Siberian silkworm, *Dendrolimus sibiricus* Tshtv. (123a).

Fungus Diseases

During the period covered by this review (*i.e.*, since 1949) a considerable number of papers dealing with entomogenous fungi have appeared. The subject matter of this literature is, however, quite varied, many of the papers being but a single report on the observation or isolation of interesting fungi. We can discuss but a few of these here.

Although interest in Entomophthorales continues to be high among insect pathologists, relatively little research has been accomplished with them during the past decade. Papers on the occurrence of various species of *Empusa* and *Entomophthora* have appeared [*e.g.* (43, 57, 81, 82, 103)], some of them including reports on the successful cultivation of certain species usually considered difficult to grow. It is clear that the significance of this group of entomogenous fungi is great from the standpoint both of their high incidence, and of their potentialities as agents of microbial control.

A new genus and species (*Zygaenobia intestinalis* Weiser) of Entomophthoreae was described by Weiser (145) who found the fungus infecting the midgut epithelium of larvae of *Zygaena carniolica* Scop., the coenocytic mycelium penetrating between the cells of the epithelium. The mononuclear conidia are formed in the gut lumen and leave the insect along with the feces. Because of the unusual pathogensis and method of distribution, Weiser placed the fungus in a separate genus, *Zygaenobia*, apparently somewhat related to *Massospora*.

Perhaps the best-known entomogenous fungus is *Beauveria bassiana* (Bals.), since the time of Bassi (1834) recognized as the cause of muscardine of the silkworm, and now known to infect a large number of other insects in nearly all parts of the world. Unfortunately, there has always been some confusion as to the taxonomic composition of the genus *Beauveria* and the relation of *B. bassiana* to other species of the genus. It is encouraging, therefore, to note the recent appearance of at least two outstanding contributions to this subject. One of these is a paper by Benham & Miranda (9), the other by MacLeod (79). The latter paper is especially noteworthy because of the thoroughness with which the author approached the problem. MacLeod studied the cultural and morphological characteristics of numerous *Beauveria* isolates. With this as a background, he reviewed the literature pertaining to 32 alleged species included in the genus *Beauveria* and the closely related, but apparently saprophytic, genus *Tritirachium*. Fourteen of these he reduced to synonymy with *B. bassiana* and *Beauveria tenella* (Delacr.) Siem.; ten he found to belong to the form genus *Tritirachium;* one he regarded as uncertain as to genus; and seven were excluded from both genera. MacLeod's conclusion that only two species, *B. bassiana* and *B. tenella*, existed among the numerous cultures he studied has far reaching significance as far as clarifying the taxonomic confusion that has heretofore plagued this group of fungi. The differentiation of these two species rests largely upon the fact that *B. bassiana* produces about 50 per cent globose spores, and *B. tennella* about 98 per cent oval spores.

B. bassiana has always appealed to insect pathologists because of its potentialities as a biological control agent. Interest from this standpoint has maintained itself in recent years and has resulted in studies of *Beauveria* infections in such insects as the European corn borer (111, 112), the codling moth (88, 111), the sod webworm (55), the imported cabbageworm (126), and others [*e.g.* (28, 80, 104, 135)].

Other Fungi Imperfecti capable of infecting insects have had recent study. Rockwood (102), for example, recorded his observations on the infectivity of *Metarrhizium brunneum* Petch and *Metarrhizium anisopliae* (Metch.) for wireworms. The latter fungus was also reported on adult rhinoceros beetles from the Palau Islands (51, 110) and India (93a), and has recently been used in experimental studies on other insects (86). A species of *Cephalosporium* was isolated from larvae of the European corn borer held in storage (8). Sussman (123) published a series of four papers concerned with the pathogenicity of *Aspergillus flavus* Link for the cecropia moth, *Platysamia cecropia* Linn. Among his interesting observations were the facts that: (*a*) *A. flavus* is capable of infecting larval, pupal, and adult stages of the insect. In the case of the pupa, infection resulted only upon injection of spores; the resistance of the pupa to infection via the integument was traced to the presence of a lipide protective layer on the epicuticle. (*b*) Six of twenty representative species of *Aspergillus* were found capable of producing a lethal infection in *P. cercropia.* (*c*) The host range of *A. flavus* was extended to in-

clude six more genera of Lepidoptera. (*d*) *A. flavus* usually infects *P. cercropia* by entering the animal through the gastrointestinal tract. It first attacks the fat body and spreads extensively through it. After consuming the fat tissue the fungus becomes established throughout the insect's body. Some phagocytosis of spores by blood cells occurs. (*e*) Diseased pupae undergo a tenfold increase in oxygen uptake as a result of infection by *A. flavus*. Weight loss (directly proportional to temperature up to 28°C.) of diseased pupae roughly parallels the respiratory increase. The weight loss is caused primarily by the evaporation of water from the spiracles.

Arachnids, as well as insects, are hosts to pathogenic fungi. *Hirsutella thompsonii* Fisher has been described (41, 42) on rust mites, and species of *Gibellula* and *Cordyceps* on spiders (84, 85). A yeastlike organism, *Acaromyces laviae* Lavie, has been found associated with the death of *Acarapis woodi* (Rennie), the mite responsible for acarine disease in the honey bee (76). *Sinella* eggs infected with *Penicillium* were observed (78) to be toxic to chiggers. Toxic reactions and death occurred in nymphs and adults that fed upon the infected eggs of the collembolan.

Since the appearance, around the turn of the century, of the monumental works of Thaxter on the order Laboulbeniales, comparatively little study has been made of this interesting group of ectoparasitic entomogenous fungi. During the past decade there have been a few papers [*e.g.* (10, 11, 107)] published relating to the systematics of the group. However, Richards & Smith (99, 100, 101) have approached the group from the viewpoint of ascertaining their morphological and physiological relation to the host insect. They found five species of *Herpomyces* to be highly but not completely host specific. The fungi grow on setae, or on hard or soft cuticle, but only on a living insect (cockroach). A tubular haustorium through the insect's cuticle was found to expand into a large bulb in the epidermal cell layer. There is good evidence that nourishment is derived from the insect host. The authors conclude that the infection of cockroaches with *Herpomyces* produces a dermatitis with a characteristic histopathology; however, there is no evidence of pathogenicity in the usual sense of the word. Reminiscent of laboulbeniacious fungi is a species of Hyphomycetes, described as *Antennopsis gallica* [Heim & Buchli (21, 62, 63)], that infects the integument of certain termites in France. The fungus makes molting of the termite difficult, hinders its movement, weakens it, and, unlike Laboulbeniales, even causes death of the host.

The study of the comparative virulence of various strains of entomogenous bacteria and fungi has not had as much attention as one might expect from the amount paid to such variations by bacteriologists and mycologists. Undoubtedly, such investigations should also be the concern of insect pathologists. In this connection, MacLeod & Heimpel (83) have pointed out that virulent strains of a pathogen might arise following genetic recombination between two or more nonvirulent strains, and that such recombinations may be expected during disease epizootics in insect populations.

Protozoan Diseases

Although many species of flagellates, amoebae, ciliates, and sporozoa are found associated with insects, the principal protozoan pathogens of insects belong to the last-named group. Members of the class Sporozoa are parasitic in habit and form spores in some stage of their development. The two most important groups of Sporozoa from the standpoint of insect microbiology and pathology are the gregarines and the microsporidia. In general, according to Garnham (49) these protozoa produce their deleterious effects in insects either by the complete obliteration of an organ, or by compression of most of the tissues in the body through sheer size or numbers of the parasite.

Gregarine infections.—Of the order Gregarinida, the suborder Eugregarinina is the larger, but the suborder Schizogregarinina includes species that are more pathogenic for insects. Most of the literature pertaining to either suborder is largely taxonomic, descriptive, and distributional in character.

Among the notable contributions during recent years to the literature on the systematics and ecology of eugregarines have been those reported as associated with Coleoptera [*e.g.* (73, 131, 132, 133, 134, 142, 143, 144)]. New species have been described, others redescribed, and interesting new data as to the host distribution of previously known species have been added. A few reports of eugregarines in other insects have also appeared [*e.g.* (74, 134a)].

One of the most frequent contributors to our knowledge of eugregarines has been Filipponi who, since 1947, has published a number of papers [*e.g.* (35, 36, 37, 38, 39)] which include greatly needed information pertaining to the basic biology, as well as systematics, or eugregarines. He studied such matters as the differential growth ratios of primites as compared with those of satellites and found them to be different. He insisted on the existence of sexual dimorphism in the polycistid gregarines. He pointed out that because trophozoites show unlimited growth, their dimensions and ratios, as commonly employed by taxonomists, have no taxonomic value. He also contributed to the taxonomy of the gregarines he studied.

Of considerable interest is the finding, by Stejskal (120), of an unidentified gregarine in the honey bee in Venezuela. According to this author the parasites attack the inner wall of the ventriculus of the adult bee, causing sickness and death, and heavy apiary losses. The unusual severity of this gregarine infection is noteworthy, as is also the supposition that cockroaches serve as vectors of the gregarine.

Our knowledge of the Schizogregarina has been significantly enhanced in recent years by the contributions of Weiser (147, 148, 150, 152, 157), especially with regard to their systematics (151, 154, 155, 156). His classification of the Schizogregarina is based on the comparative morphology of the different stages of the life-cycle of the different genera. Weiser, as well as others [*e.g.* (26)], have described new species of schizogregarines, but the total number now known in insects is not large when compared with the number of eugregarines that have been reported. Unfortunately, basic ecological and

epizootiological studies of these infections are all too few, although investigations such as Finlayson's (40) brief study of the mortality of *Laemophloeus* infected with *Mattesia* are steps in the right direction.

Microsporidian infections.—Nosema disease of the honey bee continues to be one of the most-studied of microsporidian infections. Included in the advances described in numerous papers is the treatment of the disease by drugs, such as fumagillin. However, since the diseases of the honeybee are being arbitrarily excluded from the subject matter of this review, a discussion of the matter will be forfeited.

Considerable attention, both since 1949 and before, has been paid in Czechoslovakia to microsporidian infections, notably by Weiser and his colleagues. They report such organisms as *Nosema otiorrhynichi* Weiser in *Otiorrhynchus ligustici* Linn. (105, 106, 146); *Nosema typographi* Weiser [as well as another sporozoan, *Haplosporidium typographi* Weiser (153)] in *Ips typographus* Linn. (158); *Nosema steinhausi* Weiser in the mite *Tyrophagus noxius* Zachwatkin (the first microsporidian to be reported from mites) (160); *Nosema tatrica* Weiser in *Ephemerella ignita* Poda and *Plistophora calopterygis* in *Calopteryx* spp. (161); and *Thelohania hyphantriae* Weiser in *Hyphantria cunea* Drury (149, 162, 163). The last-named microsporidian is also infectious for a number of other insect species. Incidentally, some of these as well as other species are presented by Weiser (159) in a comprehensive host list of infections in insects reported in Czechoslovakia up to 1955.

In 1953, Tanada (124) found field populations of the imported cabbageworm, *Pieris rapae* (Linn.), in Hawaii to be heavily infected (45 to 74 per cent) with *Perezia mesnili* Paillot. This microsporidian was originally discovered by Paillot in the cabbage butterfly of Europe, *Pieris brassicae* (Linn.). Tanada's was the first record of this parasite outside of France and was a new host record. Later, Tanada (125) showed that the microsporidian appears to be transmitted in nature by the ingestion of the pathogen, by transovarial passage, and by the braconid parasite *Apantales glomeratus* (Linn.). Similar studies had been conducted by Blunck (19) in Germany with *P. brassicae* in which he observed an additional microsporidian which he named *Nosema polyvora*. In 1951, another *Perezia*, *Perezia pyraustae* Paillot, was reported (54, 111, 112) for the first time in the United States in the European corn borer. Its distribution in the European corn borer included 90 counties in seven North Central states (171). A third species, *Perezia fumiferanae* Thomson, occurs in the spruce budworm in Ontario (136).

A microsporidian disease of the potato tuberworm, *Gnorimoschema operculella* (Zeller), presumably caused by *Nosema destructor* Steinhaus and Hughes, was reported on at length by Allen (1) who found it to be "a laboratory pest of considerable importance" in the mass rearing from the tuberworm of the beneficial insect parasite *Macrocentrus ancylivorus* Roh. Allen's report contains much significant data; of particular interest is his description of the effect of the microsporidian on rearing operations, and the heat treat-

ment used to control the infection in the tuberworm. As had been reported earlier by Allen and Brunson, this treatment was accomplished by placing the eggs from infected female moths in a watertight metal envelope and immersing this in a hot-water bath at 47°C. for 20 min. This procedure destroyed the protozoan but did not harm the egg.

Important evidence on one controversial matter has been furnished by Gibbs (50), working with an unidentified species of *Gurleya* found in the gut tissue of the lepidopteran *Trachea secalis* (Linn.). He found that the sporoplasm of the microsporidian is ejected from the free end of the polar filament at the time of the latter's extrusion. In this instance, at least, it does not creep out of the sporocyst as is commonly believed to be the case.

Other new species of microsporidia recently described from insects include *Nosema locustae* Canning in the African migratory locust, *Locusta migratoria migratorioides* R. & F., *Nosema buckleyi* Dissanaike in the flour beetle, *Tribolium castaneum* (Hbst.), *Plistophora melolonthae* Krieg in an unidentified species of *Melolontha*, and *Thelohania cheimatobiae* Krieg in larvae of *Cheimatobia brumata* (Linn.).

Microbial Control

Largely because of the spectacular nature of recurring instances in which microorganisms cause sharp reductions in the population of insects naturally, the use of microorganisms in the control of insect pests has been envisioned for the past 100 years or more (116). During this time enthusiasm for the possibilities of microbial control methods has waxed hot and cold, depending largely upon current instances of success or failure. Many of the attempts were made on a trial and error basis. In recent years there has been somewhat more of an effort to understand the principles involved in microbial control and in the epizootiology concerned (113). The potentialities and problems of microbial control are also in the process of being reappraised (115). Since 1949, the period covered by this review, most of the developments in microbial control have been concerned with viruses, bacteria, and fungi, with fewer attempts made to use protozoa and nematodes. Encouraging is the fact that efforts to use microbial control methods are being made in nearly all of the major agricultural areas of the world (32, 97, 130, 170).

The use of viruses.—Viruses have appeared to offer particular promise in the control of certain forest and field crop insects. Following the effective natural control of the European spruce sawfly in Canada by a polyhedrosis in the early 1940's, Canadian insect pathologists proceeded to investigate the use of viruses in the control of this and certain other sawflies. Bird has reported (18) on the use of a virus introduced from Sweden that gave effective control of the European pine sawfly, *Neodiprion sertifer* (Geoffr.), when applied in southern Ontario. According to Bird, the virus has, in general, replaced insecticides in controlling this sawfly. It has also been used successfully in Germany (46, 47, 48) and the United States (27). Similar results have been obtained in Canada using a virus against the European spruce

sawfly, *Diprion hercyniae* (17). However, a virus infective for the jack-pine sawfly, *Neodiprion americanus banksianae* Roh., was found to be impractical for control purposes because its relatively low virulence required extraordinarily heavy suspensions of it to destroy the insect easily and in large numbers (18).

Tent caterpillars are known to be susceptible to polyhedrosis viruses. There is evidence (22, 22a, 23) that such a virus may be of value in the control of the Great Basin tent caterpillar, *Malacosoma fragilis* (Stretch). On the other hand, the prospects with a polyhedrosis virus active against the forest tent caterpillar, *Malacosoma disstria* Hüb., do not appear as bright (12).

The use of viruses in the control of crop pests has been successful against such insects as the alfalfa caterpillar, *Colias philodice eurytheme* Boisduval (119), the imported cabbageworm, *Pieris rapae* (Linn.) (127), and the European cabbageworm, *Pieris brassicae* (Linn.) (16). A polyhedrosis virus has been successfully used against the alfalfa caterpillar by growers and is applicable to widescale use. In the case of the two cabbageworms, field populations have been experimentally controlled by the artificial dissemination of granulosis viruses.

Although pathogenic rickettsia-like organisms have been observed in insects [*e.g.* (30, 75, 93, 168)], no attempts to use them in the control of their hosts have been reported.

The use of bacteria.—The classic example of the use of bacteria in the control of a destructive insect is with *Bacillus popilliae* Dutky, the cause of milky disease of the Japanese beetle. Its use continues to yield effective results. Colonization of *B. popilliae* throughout the heavily infested areas of eastern parts of the United States has continued, with thousands of pounds of spore dust having been distributed (24, 96, 164). The possibility of using strains of milky disease bacteria against other insects is enhanced by the knowledge that certain other scarabs are also susceptible. For example, the larva of the European chafer, *Amphimallon majalis* (Razoum.) is susceptible to the milky disease bacteria and, according to Tashiro & White (129), there is promise of obtaining economic control of this insect through the use of these bacteria.

The control potentialities of *Bacillus thuringiensis* Berliner have received renewed interest in recent years. The promising results obtained (109) with this spore-forming bacillus in the control of the alfalfa caterpillar in California have been confirmed in subsequent unpublished experiments. Additional confirmation of its effectiveness against *Colias lesbia* Fabr. was obtained in Argentina (34). Field trials using the bacterium and certain of its close relatives against the imported cabbageworm, the diamondback moth, the caggage looper (127, 128), the European cabbageworm (15, 77, 139), and hornworms (98) have likewise yielded promising results. The use of *B. thuringiensis* was not recommended as an aid in the control of the Western grapeleaf skeletonizer (56). Recent attempts (87) in the United States to use

the organism in the control of the European corn borer have not confirmed the rather encouraging results obtained by European workers during the 1920's. While the corn borer is readily susceptible to the *Bacillus*, the American workers found in their experiments that the pathogen could not be relied on in the field, primarily because the larvae would penetrate into the plant and thus not be exposed to the spores. Of several pathogens used in greenhouse tests, Hall (55) found *B. thuringiensis* to be the most effective against the sod webworm, *Crambus bonifatellus* (Hulst). A mortality of almost 100 per cent was noted in the test populations.

In 1950 Jacobs (67) reported on the successful use of a French product known as "Sporeine" in the control of the Mediterranean flour moth, *Anagasta kühniella* (Zell.). This product, in use against vineyard pests prior to World War II, very probably consisted of sporulated *B. thuringiensis*, or a close relative. In wholemeal flour, between 0.1 and 0.3 per cent by weight of the powder completely prevented the development of infestation by newly-hatched larvae. Infestations of wholemeal flour in sheds were checked considerably by the application of surface deposits of the spores. These results are reminiscent of those obtained against this same insect by Berliner in 1915 at the time of his early work on *B. thuringiensis*. Coleopterous stored-grain insects do not appear to be sufficiently susceptible to *B. thuringiensis* to ensure its use in practical control (117).

Spore-forming bacteria infectious for the cockschafer *Melolontha melolontha* Linn. (= *M. vulgaris* Fabr.) have had recent study in Europe with the hope that they might have a place in the control of this insect (72, 163, 164). Unfortunately these bacteria have not been identified as to species, but they do not appear to be of the *B. thuringiensis* type. Significant mortalities were obtained in field tests with certain of these bacteria.

The use of fungi.—Despite the early emphasis on fungi as agents of microbial control, recent years have seen rather limited progress along this line, at least when compared to that associated with viruses and bacteria. Some of this lack of applicability is undoubtedly based on the nature of the fungi themselves, *e.g.*, on their general dependence on appropriate humidity conditions. Nevertheless, the extent to which fungi are active in natural control would indicate that their possibilities as microbial control agents are far from realized. Especially does this appear to be true with certain groups, such as the Entomophthorales, the highly parasitic nature of which indicates their potentialities as control agents. This is suggested, for example, by the important role played by species of *Entomophora* in reducing populations of the spotted alfalfa aphid, *Therioaphis maculata* (Buckton), in California (57), which instance also highlights the importance of microclimate in the efficiency of fungus infections.

Evidence that certain of the so-called friendly fungi found on scale insects in Florida do not actually parasitize the insects has been accumulating, as has the importance of the endoparasitic fungus, a species of *Myiophagus*, which causes a chytridiosis (42, 44). Practical use of the latter fungus, how-

ever, has not yet been accomplished. Certain species of *Hirsutella* may, under some circumstances, be instrumental in reducing populations of certain scale insects and mites (45, 52). The role of fungi in the natural control of certain pests in Puerto Rico has been reviewed by Wolcott (169).

Among the Fungi Imperfecti, the potentialities of the classic *Beauveria bassiana* (Bals.) and *Beauveria tenella* (Delacr.), perhaps appear the greatest as far as microbial control methods are concerned. Recent efforts in this connection, however, have yielded results similar to many of those of the past: promising but, somehow, not entirely practical. This, for example, has been the experience against such insects as the sod webworm (55), the imported cabbageworm (128), the cockchafer (68), and *Hyponomeuta* (139). Certain species of *Aspergillus* (33, 92) and *Isaria* (61) have yielded results suggesting possible control uses.

The possibility of using a yeast-like organism (*Acaromyces laviae* Lavie) in the control of *Acarapis woodi* (Rennie), a mite that parasitizes the honey bee, has been briefly tested (76), but the encouraging results need confirmation.

The use of protozoa.—Perhaps because of the relative difficulties in studying them, entomogenous protozoa have had comparatively little trial as microbial control agents. Some species, such as certain of the Microsporidia, appear to possess the virulence necessary to make them effective pathogens. Others cause rather chronic infections that may not lend themselves so readily to control uses.

Weiser & Veber (138, 162, 163) employed the microsporidian *Thelohania hyphantriae* Weiser in field tests against the fall webworm, *Hyphantria cunea* Drury. Within the first two weeks after application the incidence of infection was about 25 per cent, rising thereafter to 100 per cent infection and mortality. On the other hand, less encouraging results were obtained by Hall (55) with another microsporidian, *Nosema infesta* Hall, against the sod webworm, *Cranbus bonifatellus* (Hulst). Some degree of natural control was observed by Tanada (128) in the case of the imported cabbageworm, *Pieris rapae* (Linn.), infected with *Perezia mesnili* Paillot. Inconclusive results suggesting some promise in attempts to control the European corn borer have been obtained using *Perezia pyraustae* Paillot in the central part of the United States (149).

Of interest is the observation by Rosický (105) that infection of *Otiorrhynchus ligustici* Linn. by *Nosema otiorrhynchi* Weiser causes an accelerated effect of certain contact insecticides. Apparently the infection sensitizes the insect to doses of the insecticide that would not ordinarily kill the insect.

The use of nematodes.—Although, strictly speaking, the literature on nematodes is not to be covered by this review, it is pertinent to mention that well over a thousand species of nematodes have been reported from insects. Inasmuch as a considerable number of these are parasitic in the body cavity or the tissues of their host, the potentialities of these organisms as biological control agents would appear to be significant. Indeed, it is this reviewer's

opinion that this large group of parasites will eventually provide a significant number of effective control agents.

Although a few excursions into the use of nematodes in the control of insects have been made in the past, one of the most encouraging developments of recent years has been that based on the work of Dutky (29) in the United States Department of Agriculture. This worker found an unidentified nematode of the family Steinernematidae to be the carrier of a pathogenic bacterium which it introduces into the insect's body cavity. The bacterium not only kills the insect but also serves as food for the nematode. A large number of insects are susceptible to the nematode and the associated bacterium. Included among them are the codling moth, wax moth, imported cabbageworm, corn earworm, and more than 30 other species of insects.

Other Important Papers

Because of page limitations, it has been necessary to omit the consideration of three important groups of papers on insect pathology and microbial control. Because they are important and significant, the reader interested in a well-rounded view of the subject is urged to consult them directly. The first of these three groups is a record (97) of a symposium of 45 papers mostly concerned with the infectious diseases of insects, held in Leningrad as a joint session of the Sericulture and Apiculture, Plant Protection, and Veterinary Science sections of the All-Union Order of the Lenin Academy of Agricultural Sciences. The second group of papers is that inspired by a meeting (90) of French, German, and Yugoslav insect pathologists in February, 1956, in Darmstadt, Germany, and is included with others in Volume 1, Number 1, of the new journal, *Entomophaga*, of the Commission Internationale de Lutte Biologique (32). The third group of papers to which we are referring are those presented at the meetings of the 10th International Congress of Entomology held at Montreal, Canada, in August, 1956. Presumably these are to be published in the Proceedings of the Congress (130).

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ORAL MICROBIOLOGY[1]

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The leading theme of this review, microbial ecology of the mouth, has been so fully developed in recent years that only oblique reference is possible to the problem of dental caries, which at present is the central concern of dental research. Fortunately, however, several bibliographies, reviews and symposia are available (5, 26, 82, 108, 116, 120) which are reasonably up-to-date. The present survey is primarily concerned with the literature since Harrison's review of 1949 (39); even so, limitations of space make it impossible to report on all of the significant studies which have appeared.

Qualitative Studies of the Oral Flora

From the University of Birmingham has come an intensive qualitative study by Morris (72 to 77) of most of the oral flora in seven carious and four caries immune subjects which contributes significantly to our knowledge of oral ecology. The kinds of microorganisms identified from cultures of mouth rinses and gingival scrapings were as follows: *alpha*, *beta*, and *gamma* streptococci, enterococci, corynebacteria, actinomycetes, fusobacteria, gram-negative bacilli, lactobacilli, *Hemophilus*, *Leptotrichia*, *Nocardia*, *Streptomyces*, *Veillonella*, *Neisseria*, micrococci, and pneumococci. The viruses, protozoa, spirochetes and yeasts were not studied. Morris (77) found a considerable difference between the types of organisms observed by early workers in the oral cavity and those organisms found in healthy mouths today, apparently attributable to improved oral hygiene in recent years. From Morris' study the bacterial flora of the mouth appeared to consist in a greater degree of specialized oral parasites than had previously been believed.

Several microorganisms have been reported for the first time as members of the oral flora. Morton *et al.* (81) have detected pleuropneumonia-like organisms in the saliva of 46 persons out of 100 examined, while a subsequent study (80) of a smaller group gave an even higher incidence. These are the first reports of the organisms being present in high incidence in a region of the human body outside of the genito-urinary tract. Throat cultures yielded a lower percentage of pleuropneumonia-like organisms [Smith & Morton (114)] than mouth cultures, but as was the case for the mouth, the incidence was higher in the throat than for the normal female [Randall *et al.* (91); Klieneberger-Nobel (56)] and male [Beveridge *et al.* (10)] genito-urinary tracts. Dienes & Madoff (22) were able to obtain abundant growth of pleuropneumonia-like organisms from debris between the teeth and from material

[1] The survey of literature pertaining to this review was completed in August, 1956.

expressed from the crypts of tonsils. These workers found characteristic morphological, nutritional, and serological differences between oral and genital strains. The American strains of salivary pleuropneumonia-like organisms were isolated under aerobic conditions, but the salivary strains isolated by Edward (27) in England were obtained only under anaerobic conditions. The relationship between the aerobic and anaerobic strains is not yet clear.

The L-type phase colony is now known to develop from bacteria occurring in the oral cavity. Hijmans & Dienes (42) have demonstrated that approximately one out of four humans harbors in his mouth *alpha* hemolytic streptococci which produce L-type colonies on culture, while fewer L-type colonies develop from the throat cultures.

Massler & Macdonald (70) determined that *beta* streptococci were present in the throat in 11.6 per cent of 500 cases examined, while the organisms occurred on the gingiva in 6 per cent of the cases. Only 14 per cent of the positive cases had the streptococci on both throat and gingiva. In this regard the opinion of Duguid (25) is significant in that throat carriers are less important in creating air-borne infection than are oral and nasal carriers, since most of the expelled droplets during various expiratory activities originate from the anterior of the mouth (about the lips, front teeth, and tip of the tongue).

Williams *et al.* (128), investigating the simultaneous occurrence of enterococci, lactobacilli, and yeasts in human saliva, found that enterococci were cultivable from the salivas of 22 per cent of over 200 persons examined. When enterococci were present, high lactobacillus and low yeast counts were likely to be found in the saliva. The majority of oral yeasts are *Candida* species [Lilienthal & Goldsworthy (65)], but there have been ascosporogenous yeasts and cryptococci reported also [Fischer (31); Lilienthal & Goldsworthy (65)].

A comparison by Williams *et al.* (125) of the cultivable kinds and types of oral, pharyngeal, and nasal microorganisms indicated that the flora of the mouth and pharynx are more closely related than that of the mouth and nose or the nose and pharynx. The most varied flora of the three regions was found in the oral cavity, the next in the pharynx, and least varied was the flora of the nose. Mortimer & Watterson (79) confirmed this relationship for the pathogenic flora of the nose and throat.

The usual reports on oral ecology are almost entirely based upon salivary or mouth rinse cultures, so that the specific characteristics of various parts of the mouth remain obscure.

Attempts have been made recently (2, 30, 34) to obtain precise information about the microbial flora of the film or "plaque" on tooth surfaces by the study of appliances placed in the mouth to collect dental plaque material. Frisbie & Nuckolls (35) found that the plaque developed first as an accumulation of an amorphous mucinous material in which coccoid and filamentous organisms subsequently appear. The filamentous organisms formed a dense mat and had a characteristic parallel arrangement to one another. According

to Hurst (50), these filaments are anaerobic actinomyces. In a preliminary study, Appleman *et al.* (2) reported that the dental plaque seemed to be formed upon a matrix of squamous epithelial cells which were first attacked by micrococci and possibly streptococci; few lactobacilli were present.

According to Eggers Lura (29), dental plaque flora consists mainly of hyaluronidase-producing streptococci which depolymerize the salivary mucin, but form a mucoid substance from sucrose. A survey by Snyder *et al.* (115) of aerobic salivary organisms forming mucinous colonies uncovered only two types of bacteria capable of synthesizing mucinous polysaccharides from sucrose; one was *Streptococcus salivarius* and the other Gram-negative diplococci. However, because these mucinous polysaccharides are water-soluble, the authors believed it unlikely that they participate in the formation of dental plaques.

Krasse (62) found that *S. salivarius* occurs much less abundantly in the dental plaque than in the saliva, but no significant differences were observed between the frequency of lactobacilli in plaque and in saliva. A series of vigorous scrubbings of the tooth surface to remove as much of the plaque as possible was carried out by Sandy (99) on the assumption that the organisms of the foundation layer of the plaque would survive the scrubbing process the longest. Two Gram-positive cocci, one hemolytic and the other not, were found to persist the longest. Sandy suggested these may be the primary invaders in dental caries.

The advancing front of the initial caries lesion in the tooth enamel [Frisbie *et al.* (35)] shows but few microorganisms, and these are Gram-positive cocci alone. Onisi & Nuckolls (86) report a careful study of dentine showing the first signs of caries, in which it was found that the morphological type most often obtained resembled actinomyces. Much less frequently isolated were staphylococci, Gram-positive rods, and *Gaffkya*. Sections of the dentinal caries lesion [Prophet (90)] indicate that Tomes' fibrils are invaded first. This is followed by decalcification of dentinal tubule contents and, later, of the dentine matrix. Bacteria occupy the whole lumen of the tubule as soon as the inorganic salts are lost.

Some data are available on the microbiology of other structures of the oral cavity. According to Morris (78) the dorsal surface of the tongue almost constantly shows the presence of large masses of bacteria. He also found inflammation of the lingual tissues almost constantly present, indicating that the bacterial masses on the surface may not be entirely innocuous. In addition, Morris (78) found that guinea pigs on a vitamin C-deficient diet showed some changes of the lingual tissues, while the addition of penicillin to the deficient diet produced marked changes in the epithelium, the connective tissue, and the muscle fibers of the tongue. Since penicillin added to the normal diet produced only slight changes of the lingual epithelium, it appears that vitamin C deficiency may possibly be involved in the lingual reactions to penicillin therapy which have been frequently reported (20, 129). Only a

limited number of animals were used by Morris (78), so the results must be regarded with reservation, but the approach merits additional study.

Nash & Morrison (83) have reviewed the functions of the parotid gland, pointing out that the gland, ducts, and the excreted fluid normally are sterile. The factors they considered responsible for maintenance of sterility were (*a*) the continual secretion of saliva, and (*b*) the corkscrew configuration of Stenson's duct which hinders the retrograde flow of fluid or organisms.

The saliva from persons with active periodontal disease contains a greater percentage of epithelial cells covered with microorganisms than does saliva from patients without periodontal disease [Dreizen *et al.* (23)]. Biopsies of normal interdental gingival papillae [Schaffer (100)] show bacteria only on the surface of the epithelium, the microorganisms apparently failing to penetrate into the underlying vital tissue even when a superficial necrotizing process is present.

Quantitative Studies of the Oral Flora

Quantitative studies of the oral flora to determine total and relative numbers of the cultivable species in saliva are uncommon, since the usual reports are concerned with the effect of various diets or medicaments on salivary counts of lactobacilli and other acidogenic bacteria.

Certain problems in quantitative techniques have been recently examined. Permar *et al.* (89) studied variations in lactobacilli counts with a variety of methods designed for obtaining more homogeneous suspensions in saliva. Wide variations in counts were found regardless of the method used, but general trends could be discerned if platings were done over a period of time. Williams & Eickenberg (126) compared sonic vibration to mechanical shaking, and found that with sonic vibration higher counts of a variety of organisms were obtained from human saliva, with the exception of fusobacteria.

Ostrolenk *et al.* (87) have studied the relative reduction in bacterial numbers produced by consecutive mouth rinses with physiological salt solution. This was found to be a function of the initial bacterial counts; after the seventh wash no further reduction in count occurred. For morning samples, on blood agar, a reduction of 63 per cent was attained, while for afternoon wash series, only a 44 per cent reduction was obtained. However, variations in counts were so great that for statistically valid data a large number of subjects and repeated series of tests were required. If this was done, definite patterns evolved which merit confidence.

The studies of the Commission on Acute Respiratory Diseases of the U. S. Army (19) on the problems involved in determining the bacterial flora of the pharynx, have a direct bearing upon similar problems with the oral microflora. Of particular interest was their finding that mixtures of pure cultures failed to grow at random on being plated. The results suggested that the growth on a plate is a resultant of the nutritional interrelationships of

the organisms and the adequacy of the medium. It was apparent that the absolute or relative growth of colonies on the plate does not necessarily reflect the occurrence and distribution of bacteria in the natural habitat. The Commission suggested that the problem perhaps might be circumvented by the use of selective media.

Assuming that technical procedures are adequate, the problem still must be considered, of course, whether these relative numbers of oral microorganisms reflect a significantly stable balance or whether they are fortuitous relationships varying widely in response to every passing influence. Bibby (11), in examining stained smears from the mouth, came to the conclusion that the morphological types of microorganisms tended to maintain the same relative frequency of occurrence. A more recent opinion of Williams *et al.* (127) is that the quantitative interrelationships of the oral flora are characteristic for each person. A similar conclusion was reached by Kraus & Gaston (63) in a study of the aerobic organisms in unstimulated saliva. They found the relationships held not only for the aerobic flora as a whole, but also for certain genera and even species, such as *S. salivarius*.

Some data on the relative numbers of oral microorganisms are available from recent studies. Florestano *et al.* (32) found that *alpha* streptococci ranged from 34.5 to 50.5 per cent of the total counts of saliva plated in blood agar, while *beta* streptococci were present in much smaller numbers, i.e., 1.6 to 5.5 per cent. These figures are somewhat lower than those reported by Slanetz & Brown (111) and Ostrolenk *et al.* (87). Kraus & Gaston (63) found that *S. salivarius* averages about 50 per cent of streptococci present in saliva, and that the streptococci on the average amount to little less than half of all aerobes. In the study by Slanetz & Reynolds (112) it appears that the fusobacteria were on the order of 2,000 times less numerous in the saliva than the *alpha* streptococci, while the lactobacilli were on the order of 100 times less numerous.

The oral flora is significantly altered when certain pathologic conditions are present in the mouth. It was noted by Lammers (64) that with increasing caries activity, lactobacillus counts rise markedly, while with severe periodontal disease the flora becomes predominantly proteolytic. Fusospirochetal exudate [Rosebury (93)] has been found to have the following proportions of bacteria: 21.1 per cent spirochetes, 2.8 per cent fusiform bacilli, 2.3 per cent vibrios and other motile forms aside from spirochetes, 66.7 per cent streptococci, 4.1 per cent Gram-negative nonmotile bacilli, and 3.0 per cent Gram-positive bacilli.

Bacterial Interactions

Bacterial interactions, both inhibitory and stimulating, undoubtedly play a significant role in establishing the relative numbers of oral microorganisms. This is well illustrated by the probiotic or antibiotic effects of hemolytic staphylococcal strains from the mouth upon *Corynebacterium diphtheriae* [Berger

(8)], and by the earlier demonstration of Thompson & Shibuya (118) that the inhibitory action of saliva on the diphtheria bacillus is due to *Streptococcus mitis*.

The action of therapeutic antibiotics is also revealing. Long (66) found that with either local or systemic administration of penicillin, the sensitive oral species first disappeared, and there then followed an invasion of the mouth by Gram-negative bacilli. On stopping the antibiotic, the normal flora gradually reestablished itself. According to Carter *et al.* (16) the oral use of penicillin-bacitracin troches for one to two days results in only a transitory reduction of oral lactobacilli, lasting for periods of less than three days. The lactobacilli showed lower reductions than the streptococci, micrococci and Gram-negative bacteria, but these latter organisms required longer periods of time for regeneration of their respective populations.

Slanetz & Reynolds (112) noted in the case of two subjects that salivary counts remained essentially the same despite penicillin troches as a result of replacement of sensitive forms by a penicillin-resistant *Aerobacter aerogenes*. In the other subjects studied, extremely low counts of all the bacteria occurred during the first two days' use of penicillin troches, but counts then rose with the development of a number of new types of organisms, including yeasts and Gram-negative bacilli. From *in vitro* growth studies by Paine (88), it appears unlikely that the increase of yeasts with antibiotic therapy, so commonly observed [Kligman (57)], is attributable to stimulation of growth by the antibiotics. In Paine's opinion (88), the normal flora is primarily responsible for keeping the fungi in check.

White & Hill (124) have demonstrated *in vitro* that *Lactobacillus acidophilus* inhibits the growth of *A. aerogenes*, while Vincent *et al.* (121) have observed inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* by lactobacilli. Tomić-Karović & Nemanić (119) found antagonism by *L. acidophilus* against antibiotic-resistant *Proteus mirabilis*. Gram-positive spore-forming bacilli isolated from saliva were found by Scrivener *et al.* (106) to inhibit the growth of lactobacilli on streak plates. Attempts to inoculate antagonists into the mouth failed, so Scrivener (105) later attempted to adapt to saliva a strain of *Bacillus brevis* and of *Staphylococcus albus*, antagonistic to oral lactobacilli, with the aim of establishing them as members of the oral flora. It was found that the saliva-adapted organisms survived in the mouth several months after inoculation.

Rosebury *et al.* (98) have developed an overlapping drop plate screening method for the study and initial classification of interactive phenomena among pairs of microorganisms, and they have presented a preliminary survey of aerobic bacteria characteristic of human mucous membranes. They were able to make a provisional separation, on the basis of their data, of three interactive systems: (*a*) complete inhibition by diffusible factors on media not containing blood cells or potato juice. The effector species were streptococci or a pneumococcus, the test species micrococcus, *C. diphtheriae*

or *Neisseria catarrhalis*. This inhibition was found to be partially or wholly antagonized by the presence of blood or potato in the agar medium. (*b*) inhibition of *Candida albicans* by *Escherichia coli;* and (*c*) mutual inhibition of streptococci by different species of the same genus.

The precise mechanisms involved in these interactions are being slowly elucidated. Arvidson *et al.* (4) reported that the inhibitory action of *alpha* streptococci upon diphtheria bacilli is attributable to a diffusible factor, the action of which is blocked by red blood cells. Hegemann (41) and Thompson & Johnson (117) subsequently presented evidence that the factor is hydrogen peroxide. Wheater *et al.* (122) observed an antibiotic effect against *S. aureus* by a strain of lactobacillus, isolated from Gruyère cheese; this effect was later (123) attributed to hydrogen peroxide. Other organisms occasionally found in the saliva, particularly diphtheroids and staphylococci [Thompson & Johnson (117)], inhibit the diphtheria bacillus by mechanisms other than hydrogen peroxide production. Halbert *et al.* (38) found nine different antibiotic agents, mostly polypeptide in nature, which were produced by 22 ocular staphylococci studied. The antibiotic agents appeared to be active by at least three distinct and independent metabolic mechanisms; and indeed principally against Gram-positive species, including *Micrococcus*, *Corynebacterium*, *Sarcina*, *Streptococcus*, *Clostridium*, and *Mycobacterium*. It is significant that these antibiotic-producing staphylococci usually maintain their activity in the presence of moderate concentrations of plasma proteins or blood (37).

Physico-chemical factors appear to play a significant role in the bacterial interactions of the oral cavity. Lammers (64), in an investigation of growth curves of staphylococci, streptococci, and lactobacilli in mixed dextrose broth cultures, found that within 48 hr. the lactobacilli were present in pure culture in a medium which had fallen to pH 3.9 from pH 7.4. The addition of *E. coli* failed to influence the overpowering effect of the lactobacilli. However, when the mixture of all four organisms was placed in sugar-free medium, *E. coli* over-grew the other members of the mixture, while the medium reaction rose slightly to pH 7.8. Lammers (64) concluded that the reciprocal antagonisms between the organisms tested are decisively influenced by their degree of acid tolerance. Charlton (17) in an *in vitro* study of the role of *A. aerogenes* in the mouths of persons free from dental caries, presented evidence that its antagonism toward *Bacillus subtilis* is attributable to competition for gaseous nutrients in the medium.

An *in vitro* study by Nevin *et al.* (85) revealed that a *S. salivarius* strain had a pH optimum for growth of about 6.6, while its optimum oxidation-reduction potential was approximately −225 mv. Additional results with *Streptococcus fecalis* and *S. mitis* suggested that these findings may be valid for the entire group of viridans streptococci. Oxidation-reduction measurements by Eggers Lura (28) indicated that the Eh value of saliva from caries-active persons was significantly lower than that of saliva from caries-resistant persons. Eggers Lura pointed out this may be the result of the different

microfloras in the two groups. The streptococci, staphylococci, micrococci, corynebacteria, and neisseria seem to be the predominant flora of the caries-resistant salivas, while the lactobacilli and enterobacilli seem to be found in the caries-active salivas.

Salivary Effects on Bacteria

Lammers (64) has emphasized that *in vitro* studies of antibiotic and probiotic activities must be evaluated in relation to the interplay of a multitude of forces operating upon the oral mucosa, and it appears certain that among the *in vivo* factors of primary importance is the saliva. Caflisch (14) has written a monograph on the quite complex problem of the bactericidal action of saliva, reviewing the literature to 1946. Berger (9) in a later study attributed the inhibitory action of saliva to hydrogen peroxide from *alpha* streptococci. He lists the following factors as responsible for the maintenance of the biological balance in the living oral cavity: characteristics of the food ingested, effect of salts, mechanical cleansing, phagocytosis, action of lysozyme and "bacterionoxine," and bacterial antagonisms.

There are recent reports indicating that other factors of saliva may be active. Dawson & Blagg (21) detected in saliva from healthy individuals two antibacterial agents which showed both bacteriolytic and bacteriostatic properties against the cholera vibrio and a number of Gram-negative enteric bacilli. Saliva from cholera patients and malnourished persons showed little or no antibacterial effect. The antibacterial agents appeared to be enzymatic in nature.

No antibacterial factor in human saliva has been isolated in a form suitable for chemical study, but a beginning has been made. Kinersly & Högberg (55) have applied paper electrophoresis to the separation of protein elements in saliva, and have been able to demonstrate an antibacterial effect on *Micrococcus lysodeikticus* by a fraction which failed to pass a filter. Studies of salivary lysozyme [Joseph & Shay (51); Nemes & Wheatcroft (84)] have been almost entirely limited to demonstrating lysozyme-like activity of saliva against *M. lysodeikticus*. It is probable that lysozyme activity in the mouth is quite limited since effective inhibition of lysozyme activity is produced by pneumoccus polysaccharide, hyaluronic acid, and other acidic substances [Simmons (109); Skarnes & Watson (110)].

Smith (113) has demonstrated that salivary mucin has a virulence enhancing effect, and he has suggested that in addition to the lubricating and cleansing effects of mucin, it may very well act as a protective factor for bacteria, preventing the host's defenses from attacking the microorganisms.

Hartles & Wasdell (40) have demonstrated in saliva a water-soluble factor which enhances the glycolytic activity of the salivary flora. The factor is present in saliva collected directly from the parotid gland, and therefore is probably not bacterial in origin. It is heat stable, and dialyzable, but has not been further characterized. Hill *et al.* (44), on the other hand, have brief-

ly reported on a salivary fraction which retards the rate of acid production when added to whole saliva, and in broth stops the growth of lactobacillus. The factor occurs in larger amounts in the saliva of persons resistant to dental caries, it is thermo-stable, soluble in water but insoluble in alcohol or ether, and is not dialyzable. A later report (43) presented additional evidence to indicate that the fraction may be a polypeptide.

Studies on the nutritional requirements of oral bacteria have important implications for oral ecology. This is well illustrated by the series of studies by Koser *et al.* (60, 61) on vitamin requirements of oral lactobacilli and the vitamin levels required to attain growth and acid production to pH 5.0, the point at which enamel decalcification may occur. On extending these studies, Kauffman and co-workers (54) later found that saliva contains enough folic acid to support maximal or near maximal growth and acid production by oral lactobacilli. Salivary levels of vitamin B_6 in many cases appeared to be adequate for definite growth and acid production, and in some cases high enough to allow maximal or almost maximal growth and acid production.

Saliva may be the source of fermentable carbohydrate and amino acids for the oral microorganisms. Rogers (92) detected the disappearance of glucosamine in raw unstimulated saliva which had been incubated for from one to three days. Utilization of the glucosamine was inhibited upon the addition of phenol, thymol or penicillin to the saliva. These findings have been confirmed by Matt (71). Salivary mucin, after hydrolysis by proteolytic or amylolytic enzymes, may serve as an efficient source of essential amino acids [Dreizen *et al.* (24)] for *L. acidophilus.*

Oral Virology

Salivary effects on viruses.—Briody & Hanig (13) failed to demonstrate inhibition of influenza virus hemagglutination by human salivary mucin, but Francis & Minuse (33) shortly afterward reported success with whole saliva. It was found that the amount of inhibition varies with the individual source of saliva, and even from day to day in the same individual [Francis & Minuse (33); Knox & Still (59)]. Seltsam *et al.* (107) confirmed this report, and in addition were able to demonstrate a higher inhibitory effect upon influenza virus hemagglutination by the combined secretions of the submaxillary and sublingual glands than by the parotid secretion. They obtained additional evidence for the contention of Francis & Minuse (33) that the inhibition is based upon a reaction between inhibitor and virus. The inhibiting factor resists heating at 56°C. for 30 min. (33); about 30 per cent of its activity remains after 40 min. heating at 100°C. [Seltsam *et al.* (107)]. The probability that the inhibitory factor is a mucoprotein was suggested by Seltsam *et al.* (107). McCrea (67) has described the preparation, from submandibular glands of sheep, of mucoid with high antihemagglutinin activity against influenza B virus and moderate activity against influenza A virus.

Apparently human saliva contains more than one agent inhibitory for

viral hemagglutination. Blood group A substance, which occurs in saliva, has considerable inhibitory effect upon influenza virus hemagglutination [Green & Wooley (36)]. Jungeblut *et al.* (52) have reported that some samples of saliva may inhibit Columbia SK virus hemagglutination but not influenza virus hemagglutination. In this case it appears that the inhibitor acts on the red cells rather than the virus, is heat labile, and does not pass ordinary dialysis membranes. A later study [Jungeblut & Knox (53)] indicated that the active principle is an enzyme which appeared closely related to receptor-destroying enzyme (RDE) of bacterial origin. However, it was not possible to isolate RDE or a similar principle from the mixed oral flora of one positive reactor investigated. It is of interest that the active principle was present more frequently and more consistently in the saliva of acute and convalescent poliomyelitis patients than in the saliva of healthy individuals [Jungeblut & Knox (53); Hofman (45)].

In mice, inoculation of herpes simplex virus plus gastric mucin into the foot pad, the cerebrum, or the loose tissue of the groin results in failure of the virus to infect, as demonstrated by Armstrong (3). He considered the protective effect attributable to a simple ensnarement of virus particles by the mucin so that they could not approach susceptible cells.

Bacterial effects on viruses.—Knox (58) has claimed that salivary mucolytic enzymes of bacterial origin are present in saliva and this suggests that if there is a protective effect of salivary mucin against herpes simplex virus, it may be destroyed by oral bacteria.

On the other hand, a bacterial mechanism in oral resistance to viral infection is suggested by the study of Horsfall & McCarty (48) in which infection of mice with the pneumonia virus of mice was found to be markedly lessened by the intranasal instillation of Streptococcus MG, *S. salivarius* type II, or a number of other bacterial species, or by the intranasal instillation of certain polysaccharide preparations from Streptococcus MG, Friedländer's bacillus, and other bacteria. The effect did not seem to be caused by a blockade of virus receptors on the cells of the respiratory tract mucosa.

Acid and alkaline tissue phosphatases have been shown by Amos (1) to have a considerable inactivating effect upon herpes simplex virus. Since bacterial and tissue phosphatases are present in the saliva (15), it is possible that they contribute to the control of herpes simplex virus in the mouth. Béquignon *et al.* (7) have demonstrated that fixed rabies virus mixed with normal canine slaver is virulent for the guinea pig by the subcutaneous route, whereas virus alone is not. The effect could be repeated (6) by substituting testicular hyaluronidase for the slaver.

Bacterial Associations in Oral Infections

Pathogenic bacterial associations in the mouth involve especially the periodontium, but apparently such associations may also involve deeper tissues, as in actinomycosis (46, 47). Bibby (12) has separated periodontal dis-

eases into three divisions for bacteriological considerations: (*a*) a group characterized by fusospirochetal gingivitis, (*b*) marginal giginvitis, in which no specific bacterial types predominate, and (*c*) the more advanced states of periodontal disease, constituting periodontitis. According to Rosebury (94) these diseases proceed through a characteristic mixed infection, apparently qualitatively similar in all clinical types of periodontal disease, but differing in degree and in acuity of inflammation and associated damage. Infection is clearly not primary.

Vincent's infection.—Rosebury *et al.* (95, 96, 97) have carried out studies to determine the part played by the components of the bacterial mixture found associated with fusospirochetal infections. Fifteen anaerobic bacteria isolated from various sources, and including spirochetes, fusobacteria, vibrios, streptococci, a *Bacteroides*, and a presumptive *Leptotrichia*, were inoculated subcutaneously into guinea pigs individually and in various combinations (95). In no case was it possible to reproduce the lesions obtained with exudate from Vincent's infection. Inclusion of sterile filtrate of Vincent's disease exudate as the suspending medium for cultures isolated from a single source, and use of the same proportions of the individual members of the mixture as that observed in the exudate (97), again failed to yield the typical fusospirochetal response. Further attempts (96) at analysis of another infective exudate failed to demonstrate a virus, while additional isolations to give a mixture of 34 organisms still failed to produce a characteristic lesion. The possibilities remained, however, that all the essential organisms were not cultivated, or that there occurred loss of viability of certain strains during the process of recombination.

Later, Macdonald *et al.* (68) obtained a set of 17 distinctive bacterial strains, including a spirochete, from infective exudate; upon recombination these produced typical transmissible fusospirochetal infection in guinea pigs. Success apparently was attributable to the development of a new technique for recombination of the individual bacteria which was designed to prevent loss of viability of the individual strains. Macdonald *et al.* (69) were able to determine that the minimal combination capable of producing typical guinea pig "fusospirochetal" infections included only four organisms: two strains of *Bacteroides*, including a strain of *Bacteroides nigrescens*, a motile gram-negative anaerobic rod and a diphtheroid.

Analyses of the mechanisms involved in this pathogenic bacterial association are at present barely started. Hunter & Macdonald (49) were unable to demonstrate soluble toxic substances either in suspensions of scrapings from periodontal disease in humans or in guinea pig fusospirochetal exudate.

Marginal gingivitis and periodontitis.—As Bibby (12) has emphasized, the effects on the tissue of the bacteria associated with periodontitis are a matter of some uncertainty; nevertheless he was of the opinion that bacterial products are in large measure responsible for the destructive processes. A series of papers by Schultz-Haudt and co-workers have since appeared to

substantiate Bibby's view. Bacteria-free filtrates prepared from bacteria isolated from the gingival margin of cases of gingivitis were shown (102) to have an hyaluronidase-like effect in rabbit skin. Subsequently, Schultz-Haudt *et al.* (101) demonstrated that several microorganisms isolated from gingival pockets of patients with gingivitis were able to produce hyaluronidase and collagenase. Collagenolytic activity was found by Schultz-Haudt & Scherp (103) in a study of mixed cultures of gingival bacteria from 13 of 19 persons with chronic marginal gingivitis, but only 3 of 21 persons with clinically normal gingivae showed such activity.

In a further investigation of the bacterial mechanisms in gingival disease, Schultz-Haudt & Scherp (104) found that phenolsulfatase, which may act on the chondroitin sulfate of tissues, is a product of interactions between a fusiform bacillus and various other gingival microorganisms, especially staphylococci and microaerophilic Gram-negative cocci. Apparently the two latter ancillary organisms lower the oxidation-reduction potential to the point required for growth of the fusiform bacillus, and provide accessory growth factors. Phenolsulfatase has been found in the saliva (18), but it still remains to be demonstrated whether the enzyme plays a role in the pathogenesis of either Vincent's infection or periodontitis.

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NONGENETIC EFFECTS OF RADIATION ON MICROORGANISMS[1,2]

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INTRODUCTION

A precise definition of the material to be included in this review is difficult to make. Nongenetic effects might be defined as those which are not inherited; those which do not involve changes, temporary or permanent, in genetic material; or as those which have been studied from points of view other than genetics. None of these definitions is really satisfactory in practice, and a somewhat variable and arbitrary combination has been used. In most instances, the inclusion of possible nongenetic effects has been emphasized rather than the exclusion of those that might be genetic. Space considerations have prevented an adequate review of the extensive literature on the effects of radiation on viruses, including the induction of lysogeny. Some of this work might properly have been cited, but it has seemed better to omit it entirely rather than give a brief and inadequate review. Moreover, the point of view in much of the virus work has been quite different from that in the work which has been included.

Work on microorganisms through about 1951 was covered exhaustively in several chapters in Hollaender's "Radiation Biology" (89). Bacq & Alexander's book (9), reviews by Sparrow & Forro (176) and by Mortimer & Beam (145), the reports of the *National Academy of Sciences—National Research Council* (155, 156), and a *Ciba Foundation Symposium* (209) provide more recent material on both microorganisms and higher forms.

PHYSIOLOGICAL EFFECTS

Inactivation and Death

Inactivation of microorganisms, except protozoa, is usually measured by plating and determining the fraction of the original cells that develops colonies. Since it is impossible to say whether the cells have died or simply failed to multiply, the word "inactivation" rather than "death" is properly used. The word "death" can be used for protozoa which are followed individually after irradiation.

Fungi.—The evidence that inactivation of *Neurospora* conidia and yeast cells involves nuclei rests on the close relation between the number of nuclei per conidium or ploidy of the yeasts and the inactivation kinetics.

[1] The survey of the literature pertaining to this review was completed in September, 1956.

[2] Abbreviations used in this paper: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; ATP, adenosine triphosphate.

[3] Operated by Union Carbide Nuclear Company for the U. S. Atomic Energy Commission.

Much of the work with yeasts has been interpreted in terms of dominant and recessive lethals (144); however, there are difficulties with certain aspects of this interpretation (137, 208). Yeasts are probably inactivated by damage to nuclei but the nature of this damage is not yet adequately understood. In *Neurospora*, inactivation involves the whole nuclei (7, 8, 148), but there is also evidence that interaction of nuclei can lead to reversal of otherwise lethal damage. Here, again, the nature of nuclear damage is not yet well defined; but, since most of the workers with fungi have treated inactivation from an essentially genetic point of view, this work will not be reviewed in detail.

Bacteria.—Zelle & Hollaender (214, 215) have pointed out that the evidence on the role of lethal mutations in inactivation of bacteria is conflicting. Probably inactivation results from several kinds of events which may vary widely in importance from case to case. It is unnecessary to reiterate the points made by these reviewers; the next few paragraphs should be considered more as an addendum to their discussions than a complete summary of the current status of the problem. Possible nongenetic causes of inactivation are intentionally emphasized.

Some additional information about the form of the inactivation curves has been obtained. Comparative curves for several different microorganisms have been published for x-rays (73) and most are of a simple form. The inactivation curves for *Escherichia coli* B/r have been obtained (181) for various stages of the growth cycle. The curves increase in multiplicity simultaneously with increase in the number of nucleoids, but the possibility that the bacteria are multicellular instead of multinuclear makes interpretation difficult.

There have been additional instances in which the responses of mutation and inactivation to modifying conditions have been compared. In *Streptomyces*, a number of posttreatment agents produce effects on ultraviolet-induced mutation which differ from those on inactivation (197, 198, 200). Continuously irradiated *E. coli* show no evidence of death during the log phase of growth although the rate of mutation continues unchanged (169). On the other hand, striking parallels between the effects of modifying agents on mutation and inactivation have been reported for x-irradiated *E. coli* (95). Zelle (214) has pointed out that nonparallel behavior of inactivation and some kinds of mutation proves little since mutations differ considerably among themselves. Parallels between mutation and inactivation are not strong evidence that the latter is caused by lethal mutation as long as it remains possible that both are secondary consequences of similar primary events.

Two nonmutational hypotheses for inactivation have received some attention and support: that inactivation results from damage to the enzyme-synthesizing or to the DNA-synthesizing mechanism. There are reasons why both effects might be attributed to nuclear damage, perhaps quite specific and localized nuclear damage but, since both are apparently readily reversible, provisional treatment as nonmutational changes is justifiable.

Ultraviolet light inhibited the ability of *Aerobacter aerogenes* to utilize certain adaptive substrates as the sole carbon source (147, 166). When irradiated bacteria were put on such adaptive carbon sources the kinetics of the inactivation were simple and had a multiplicity of 1.7, corresponding to the average number of nuclei. Preadaptation to the substrate allowed the irradiated bacteria to utilize it; stable nutritional mutants were excluded. The effect showed a certain specificity since bacteria unable to adapt to one substrate were, in some instances, able to adapt to another, but there were certain correlations between the losses. Norman has referred to these changes as "phenocopies."

Stapleton, Sbarra & Hollaender (186) found that *E. coli* B/r, grown on complex medium, x-irradiated, and then plated on minimal medium, showed more inactivation than if plated on complex or supplemented minimal medium. Bacteria grown on minimal were much less influenced by the plating medium. They suggest as a possible explanation that cells grown on complex medium lack certain enzymes needed for growth on minimal and that radiation inhibits the ability to form these enzymes. The supplements might, then, aid the cells to recover the ability to synthesize the necessary enzymes. Stable, nutritionally-deficient mutants were not involved, since none could be isolated from the survivors on supplemented medium.

These two cases give considerable grounds for thinking that inactivation can result from a temporary alteration in the cells' ability to synthesize certain adaptive enzymes. Just as for mutations and inactivation, cases of nonparallel behavior between effects on adaptive enzyme formation and inactivation have been reported (72, 109).

Inhibition of DNA synthesis has been suggested by a number of investigators as a major pathway for radiation damage to cells. Kelner, especially, advocated this hypothesis for ultraviolet-irradiated bacteria (113, 114, 115) and suggested that inactivation and mutation are secondary consequences of the effect on DNA synthesis. Normally, the block in DNA synthesis after ultraviolet is temporary (104), but irreversible inactivation has been reported of a thymine-deficient mutant of *E. coli* as a result of inhibition of DNA synthesis by omission of thymine from the medium (14, 34). DNA synthesis was blocked while RNA and protein synthesis continued, and it was suggested that the resulting imbalance between nucleus and cytoplasm led to inactivation. Two types of inactivation were distinguished when ultraviolet-irradiated *E. coli* were held for various times in liquid medium before plating. One of them showed a quantitative parallel with the inactivation slope for the unirradiated mutant in deficient medium, and was attributed to inhibition of DNA synthesis by the radiation.

Thus, there is appreciable evidence for inactivation of bacteria through temporary inhibition of enzyme and DNA synthesis. The temporary nature of the inhibitions, when inactivation is avoided, demonstrates that stable mutations blocking these syntheses are not involved. The simple form of the inactivation curves and the relation to nuclear number suggest that the effects may eventually be explained by specific but reversible damage to the

genetic system of the nucleus, but this is by no means certain since a cytoplasmic basis for adaptive enzyme formation has been postulated (177). It also remains possible that inactivation is not a direct consequence of the inhibited syntheses but that the latter give reversible lethal mutations, if such occur, a chance to stabilize. However, this adds another step that is not presently required by the evidence. In any case, it can be demonstrated that part of the inactivation results from easily reversible steps closely associated with inhibition of the synthesis of enzymes and DNA. A priori, it would be expected that stable lethal mutations would also contribute to inactivation, but the relative magnitude of this contribution cannot be assessed at present and probably varies under different circumstances.

Protozoa.—As pointed out in the introductory paragraph to this section, the word "death" is properly used for protozoa because individual protozoa are followed and their death or survival is determined directly. The main features of radiation-induced death in these organisms have been reviewed (117, 206), and only brief comment is needed here.

In the ciliates, death (vegetative death) before autogamy or conjugation is almost certainly not the result of ordinary lethal mutation, because the highly polyploid macronucleus should prevent the expression of such mutations. Moreover, vegetative lethality parallels reversible division delay, not lethal mutation, in its dose response and modification by various agents (118, 119, 159). Because of these parallels, it is more convenient to consider vegetative death in the next section.

Death after the sexual processes of autogamy and conjugation has been shown to be caused by recessive or dominant lethal mutation (117, 124). In addition, there are inherited nuclear effects of unknown basis that lead to abnormal nuclear behavior at conjugation and, presumably, autogamy (51, 124). Probably lethal mutation and abnormal nuclear behavior together can account for the various peculiarities of postautogamous lethality pointed out by Powers & Ehret (159); and it seems unnecessary, as yet, to invoke other hypotheses to explain them.

Death in rhizopod protozoa is also more easily discussed in conjunction with division delay in the next section. However, it should be mentioned that Friedrich-Freska & Kaudewitz (60) have interpreted delayed death in *Amoeba proteus*, after incorporation of P^{32}, as segregation of lethal mutations from originally multistranded chromosomes. The number of strands (16) required by this hypothesis seems rather large.

Growth and Division

Bacteria.—It has long been known that several strains of *E. coli* form long filaments after x- and ultraviolet irradiation (54, 113, 215). This shows that division is more readily inhibited than growth, as is also shown by the increase in the ratio of dry weight to cell number (179).

Cytological studies with both the electron and light microscope have now given a fairly complete picture of the visible changes in cells and nucleoids at various doses of x- and ultraviolet radiation. At doses from which appreci-

able survival occurs, the nucleoids fuse (78) into one or two central masses with some increase in cell length but no apparent increase in chromatin. This may correspond to the period of inhibition of DNA synthesis. These masses elongate, and the amount of chromatin increases and becomes fragmented (78, 110, 111, 112) while the cell elongates considerably. Eventually, typical nucleoids are cut off from the ends of the elongate mass and incorporated in new cells (78, 112). At higher doses, the elongate central chromatin mass may partially disintegrate (78) or become vesicular. Presumably, these latter changes are irreversibly lethal. Cortelyou *et al.* (36) report disintegration of the chromatin in living bacteria and, from repeated observations of the same field, were able to show that the majority of bacteria exposed to a 99 per cent lethal dose of ultraviolet do not divide at all or, if they have started division before irradiation, do not complete it.

It has been reported that the lag phase of growth is relatively easy to lengthen by x-rays (17, 169). No effect on rate of growth during the log phase was found by turbidity measurements (169) or by successive platings (17).

Yeast.—The budding of *Saccharomyces cerevisiae*, just as the division of *E. coli*, can be inhibited by doses of x-rays which allow increase in size and protein content to continue for a time (178, 179). Sarachek *et al.* (170), on the basis of curve analysis, distinguish an effect on budding at low doses of x-ray that is thought to be independent of inactivation, and an effect at high doses that is associated with inactivation. Burns (31) finds that x-irradiation of fully grown cells has little effect on the time of the next budding but delays the following one. Irradiation of a budding cell delays the generation of budding after the one irradiated, and the two daughter cells, which had a common nucleus at the time of irradiation, show a correlation in the amount of the effect. Thus, in yeast, unlike *E. coli*, the effect on division is not immediate but is delayed. Townsend *et al.* (195) find that doses of ultraviolet which delay budding also delay the formation of the crescent of "centrochromatin" before budding; but doses of x-rays that delay budding do not.

Extensive cytological observations on a number of yeasts after x-irradiation have shown a variety of changes in the cytoplasm and its granules (141). Somewhat similar observations have been made on the mold, *Allomyces javanicus*, exposed to gamma radiation (167).

Ciliates.—In ciliates, unlike bacteria and yeasts, the division of the cell is no more sensitive to radiation than is the growth process. In fact, some of the evidence suggests that the effect on growth may be primary, with division delay a secondary consequence. However, synchronized cultures of *Tetrahymena* show about the same delay in the first division as unsynchronized ones (46), which suggests a more specific block in the division process since animals in synchronized cultures are already quite large. A specific block is also suggested by the observation that animals irradiated while dividing complete the process more slowly than usual (205).

The delay in division is not usually confined to the first division but extends over several divisions before eventual complete recovery (64, 117); various patterns of this delay have been found. In some cases, the delay con-

sists of two effects having maxima during the first and at some later division interval (33, 125). The two effects are partially separable and respond differently to modifying agents (120, 125). In other cases, the maximal effect is on the first division with progressively lesser effects on later ones (29, 66, 88, 158). It should be pointed out that the two patterns are not always easily distinguished; the second may be only a variant of the first with the late effect shifted backward upon the early one.

Death may occur only before the first division, during the secondary delay (125), or after a number of divisions in the absence of a secondary decline in division rate (29). The relation between death and division delay is clear in certain cases and, in general, it seems probable that death is often an extreme result of the disturbance which leads to division delay. On the other hand, rapid death after very high doses probably results from more extensive disruptions of cellular organization. The appearances are much as would be expected if membranes were disrupted, leading to dislocations of water and other materials.

Attempts to localize the effects on division to the nucleus or cytoplasm have not provided a definite answer. Giese (64) and his associates have interpreted division delay as a nuclear effect because of the nucleoprotein-type action spectrum, but it has been pointed out that cytoplasmic nucleoproteins could just as easily be implicated (117). Brandt & Giese (27) report that a cytoplasmic effect, immobilization, is not photoreversible whereas division delay is; and this, in conjunction with evidence from other organisms, was taken to mean that division delay is nuclear. But, since x-ray-induced division delay responds to modifying agents in a manner entirely different from that of lethal mutations (118), the reverse conclusion could be drawn. It seems probable that division delay induced by x-rays and ultraviolet is basically the same phenomenon (125); the results with modifying agents would therefore, in a sense, cancel each other out. Powers (158) suggested a self-reproducing cytoplasmic particle model for x-ray-induced division delay in *Paramecium aurelia;* but it is pointed out in the discussion of Powers' paper that the model will not fit, without extensive alterations, the evidence that division delay in this species consists of two partially separable parts. Powers & Ehret (159) point out that lethal mutations and division delay are produced by about the same doses of ultraviolet or nitrogen mustard whereas much higher doses of x-rays are required to produce division delay than mutation. These relations would be understandable if division delay were the result of injury to the superficial cytoplasm, since ultraviolet and nitrogen mustard would be expected to penetrate relatively poorly to the nucleus. This last argument seems to the reviewer to be the only one offering any real strength, and even it is not conclusive.

The work with the ciliates throws doubt on the generality of the hypothesis that inhibition of DNA synthesis is a primary cause of genetic as well as other radiation damage. In the first place, Ducoff (46) has shown that the x-ray-induced block to division in *Tetrahymena* occurs in synchronized cultures. In these cultures, DNA is already present in several times the normal

amount and is segregated out without new synthesis over the course of several rapidly succeeding divisions. Consequently, a block in the synthesis of this compound cannot be implicated in the division delay. More important, perhaps, is the finding that micronuclear and macronuclear division, and therefore presumably DNA synthesis, continue at nearly the normal rate (62) even in animals in which large amounts of mutational damage have been done to the chromosomes by x-rays. In fact, the evidence in *Paramceium* would be more in keeping with the idea that inhibition of DNA synthesis (as measured indirectly by division delay) is a secondary consequence of damage to some mechanism controlling the onset of division or the maintenance of growth.

Finally, three miscellaneous observations on division and survival in the ciliates may be mentioned. Recovery from division delay after 80 kr of x-rays was so complete in *P. aurelia* that the animals even aged (progressively declined in division rate) at the same rate as unirradiated controls (123). On the other hand, the aging of resting cysts of *Colpoda maupasi* was accelerated by 100 kr of x-rays (154). It has been reported that chronic irradiation of *Paramecium* at 4 to 8 r per hr. accelerated division (37), but since it is very difficult to control the environment in such experiments well enough to make the controls and treated really comparable, more extensive work would be required to establish this unexpected effect beyond question.

Rhizopods.—Both division delay and survival have been studied with the genera *Amoeba* (uninucleate) and *Pelomyxa* (multinucleate). For *Amoeba*, the maximum delay appears in the first division after x-irradiation, but in the second after application of nitrogen mustard (151, 152, 153). After x-irradiation, the amoebae may survive three to six weeks without division before eventually dying (153). After a lethal dose of ultraviolet, amoebae may survive as long as 20 to 30 days without divison (138). Maxima of death after 5 and 31 divisions have been reported (60) for internally incorporated P^{32}. *Pelomyxa illinoisensis* survives three to seven days, sometimes with an intervening division, and then dies with an LD_{50} of only 10 kr (42). *Pelomyxa carolinensis* and *P. illinoisensis* die within one day with LD_{50}'s of about 100 kr; but *P. carolinensis* does not show delayed death at lower doses. Obviously, there are appreciable species differences in the details of the response to radiation.

Both *Amoeba* and *Pelomyxa* have been used for experiments to ascertain the part of the cell that is damaged by the radiation. The most direct evidence comes from nuclear transplantation experiments with *Amoeba* (153). Nuclei were transferred from irradiated or unirradiated amoebae to irradiated or unirradiated enucleate cytoplasm. The LD_{50} for nuclear death was about 120 kr whereas that for cytoplasmic death was 290 kr. The relations are such that a dose just sufficient to kill nearly all amoebae by nuclear death would kill few if any by cytoplasmic death. Death from nuclear damage occurred without division within three to six weeks, whereas death from cytoplasmic damage occurred within three days. Doses below 280 kr produced reversible damage to the cytoplasm—stickiness, tendency to burst when

touched, inability to move and feed, and delay in division in the presence of an unirradiated nucleus. Such reversibly damaged cytoplasm could produce nuclear injuries, as shown by experiments in which an unirradiated nucleus was transferred into damaged cytoplasm and out again into normal cytoplasm.

Mazia & Hirshfield (138) compared the sensitivity to ultraviolet of whole, nucleate halves, and enucleate halves of *A. proteus*. The nucleate halves were more sensitive than whole animals, suggesting a role of the cytoplasm at least in some recovery process. The lifetime of the enucleated halves could be appreciably decreased by doses somewhat less than the LD_{50} for nucleate halves, demonstrating that the radiation does have an appreciable effect on the cytoplasm in this dose range.

A series of papers by Daniels (39 to 43) on the multinucleate genus *Pelomyxa* shows that the addition by fusion or injection of untreated cytoplasm to treated pelomyxae considerably reduces the effects of x-rays and nitrogen mustard. Use of centrifuged fragments shows that unirradiated nuclei are not required and suggests that mitochondria are involved. Nuclear division is synchronous in *Pelomyxa*, and the synchrony is maintained whether or not a considerable number of unirradiated nuclei are added. This indicates that delay in nuclear division is not the result of autonomous damage to the mitotic mechanism of individual nuclei, but is the result of damage to the whole organism.

All of the evidence found with the rhizopods agrees in demonstrating an important role of the cytoplasm in the processes leading from radiation damage to death and division delay. The evidence from the transplantation experiments with *Amoeba* shows that the nucleus is appreciably more sensitive than is the cytoplasm in this genus; but the evidence from *Pelomyxa* could be interpreted in terms of cytoplasmic damage only, although a critical test has not been made for nuclear damage that is repaired by unirradiated cytoplasm.

Miscellaneous

Cytoplasmic entities.—Radiation might cause reversible cellular damage by inactivating part of some class of self-reproducing particles, as postulated by Powers (158) for division delay in *Paramecium*. Actually, few examples of this mechanism have been found so far, but a model for it exists in the kappa particle of *Paramecium* (117). The inactivation by radiation of this particle and of the related entity, paramecin, has been the subject of recent studies concerned with kinetics (146), action spectra (174), and modification (63, 118). Studies on the production of petites in yeast may also be mentioned (165).

Hanson's studies (75) with an ultraviolet microbeam on the gullet of *Paramecium* provide another model of radiation action, this time on a cytoplasmic entity which duplicates itself at division. The injured gullet either fails to form a new gullet, forms an abnormal one or, if the injury is not too severe, forms a normal one.

Conjugation.—After high doses of x- and ultraviolet radiation, the mating reactivity of *Paramecium* is decreased; but it is increased at low doses (49, 206). Ehret (48) has carried out extensive studies on the photoperiodism of the mating reaction in *Paramecium bursaria.*

BIOCHEMICAL EFFECTS

This section is concerned with those effects of radiation which are detected by biochemical methods. Of course, the primary radiation lesion to the cell must, in the final analysis, be a biochemical change but not necessarily one that can be detected by present biochemical procedures. Nonetheless, the hope is always present that a primary lesion will be found by such methods. The chances of finding a primary change are essentially nil if the biochemical test is not performed until many hours have passed, or if cellular disintegration has occurred. Even if the test is made immediately after irradiation, there are still many difficulties found in tracing causal relations. These difficulties are probably no greater than those encountered with the biological criteria of damage; but the urge to make simple interpretations in terms of radiation chemistry, is probably greater. Despite difficulties in interpretation, the information about the biochemically detectable changes, especially those immediately after irradiation, is extremely important for a description and understanding of the damage.

Appreciable progress has been made in this area of radiation biology in the last few years. The reviews of Errera (56) and Bacq & Alexander (9), as well as the proceedings of conferences on the biochemical aspects of radiobiology (155) and on ionizing radiations and cell metabolism (209) may be consulted for more details and for work with other organisms.

Inhibition of DNA synthesis.—There has been considerable agreement that DNA synthesis is inhibited by relatively low doses of radiation [reviewed in reference (9)] in microorganisms (except the ciliates) and in higher organisms. In *E. coli*, at lower doses at least, this inhibition takes the form of a reversible block in synthesis after both ultraviolet (54, 113) and x-irradiation (44). Supporting evidence for a block in DNA synthesis after ultraviolet has been obtained from comparison of ultraviolet-treated *E. coli* with those in which DNA synthesis was blocked by omission of thymine from the medium (14, 34). Meissel (141) reports that uptake of P^{32} into DNA in yeasts is more sensitive to x-rays than are most other biochemical effects.

The block in ultraviolet-irradiated *E. coli* occurs in a late step in the synthesis since the percursors of DNA, but not to any extent those of RNA, accumulate in irradiated cells (100, 101, 102, 104, 105). The block is apparently associated with a disturbance in anaerobic phosphorylation (100, 106) which recalls Brachet's findings (26) that anaerobic phosphorylations are disturbed in enucleated cells.

It has been suggested (105) that the block is the result of interference with replication through an alteration in the DNA or deoxyribonucleoprotein. This idea receives some support from the observation (128, 129) that *E. coli*, given doses of x-rays or ultraviolet sufficient to block synthesis of bac-

terial DNA, is still capable of supporting phage development when unirradiated phage (uninjured DNA) is introduced. In opposition to this is the evidence cited in a previous section that ciliates can continue to synthesize nuclear material (presumably DNA) at nearly normal rates even after doses which cause extensive mutational damage. Thus, this kind of damage, at least, is not necessarily associated with a delay in synthesis. It is also of interest that DNA *in vivo* has been reported to be not especially sensitive to ionizing radiation as judged by the physical properties of the extracted material (149), although DNA *in vitro* is quite sensitive (5). The possibility that the observed inhibition in DNA synthesis is a secondary consequence of mitotic inhibition has been raised by Howard (97), and Gaulden (61) has shown that mitotic inhibition can be produced in stages in which no DNA synthesis occurs. Thus, the relation of the observed inhibition of DNA synthesis to other radiation effects, especially to injury of the DNA itself, has not been clearly established and it is possible that the situation is different for ultraviolet than for x-rays. Further discussion of these problems is contained in the general references cited at the beginning of this section.

Adaptive enzyme formation and protein synthesis.—Another function which seems to be blocked fairly easily by radiation is the formation of adaptive enzymes (22, 34, 109, 147, 166, 193, 194). There are, however, cases in which adaptive enzyme formation does not seem to be especially sensitive (177, 213); and it seems possible that different enzymes differ considerably in this respect. In one case, the ability to form different adaptive systems has been shown to be separately inactivated although there are some correlations between different enzymes that are not entirely explained (147). The occurrence of specific blocking and the apparent connection between the kinetics of inhibition and the number of nuclei has led Norman (147) to the conclusion that the inhibitions are specific nuclear effects.

A specific inhibition of the synthesis of certain enzymes is also more compatible with the evidence that over-all RNA and protein synthesis is not as sensitive to irradiation as DNA synthesis (104, 105); it is also consistent with evidence for yeast (178) that doses which inhibit division but allow some increase in size, cause increases in the frequency of some amino acids and decreases in the frequency of others. Thus, there is evidence that certain specific protein syntheses rather than protein synthesis in general are inhibited at low doses, but other evidence for yeast (141) suggests that even at fairly low doses there is some inhibition of RNA and protein synthesis, so that over-all effects on protein synthesis as well as specific effects may possibly occur within the same dose range.

The inhibition of penicillinase in *Bacillus cereus* by ultraviolet has been investigated at various times after induction, and the hypothesis is advanced that radiation blocks the formation of new enzyme-forming centers rather than the formation of enzyme by pre-existing centers (194).

Other effects on enzymes.—A number of enzymes and enzyme systems have been investigated. In general, most of these are not easily affected (56) although the possibility remains that this is because the methods for detec-

tion of effects are not very sensitive. For example, there have been a number of studies of respiration (50, 81, 132, 141, 143, 168) most of which, although not all, reveal little effect except at fairly high doses. It has been shown for x-irradiated *E. coli* (23) that respiration is inhibited following irradiation only after an intervening period of incubation; the suggestion was made that enzyme replacement rather than direct inactivation of the enzyme system was involved.

A variety of other enzyme systems and enzymes have been studied both *in vitro* and *in vivo* after irradiation (1, 50, 81, 87, 98, 141, 161, 163, 168), but no general conclusions seem possible as yet except that most of these systems are not very sensitive. An increase in catalase activity has been reported after x- and gamma irradiation of yeast and attributed to alteration of the physical state of the enzyme (6); but it is not clear that breakdown of the cell membrane with consequent increase in apparent activity is excluded.

There have been two recent attempts to calculate the sensitive volumes of intracellular substances by irradiation *in vivo* and measurement of activity in dried preparations (99, 157). In both cases, the sensitive volume is larger than the molecular volume, indicating some diffusion of radiation products from the immediate vicinity of the substance.

Adenosine triphosphate metabolism, membrane effects.—Kanazir & Errera (100, 103, 106) find only a minimal effect of ultraviolet irradiation on aerobic but a marked effect on anaerobic ATP formation in *E. coli.* They associate this result with the block in DNA synthesis and with Brachet's finding (26) that anaerobic phosphorylations are disturbed in enucleate cells.

Billen *et al.* (24) find that *E. coli* B/r exposed to 60 kr of x-rays continues to produce ATP at about control rate, but that much of it is released into the medium after a lag. Billen & Volkin (25) report the disappearance after 120 kr and 90 min. incubation of particles capable of *in vitro* oxidative phosphorylation. A highly polymerized form of DNA also disappears. The particles do not bind ATP in normal cells so that their disappearance cannot account for the release of ATP into the medium, and alterations in membrane permeability are suggested.

Bair and his associates (11, 12, 13) have reported disturbances in bakers' yeast in potassium exchange with the medium after 90 kr of radiation. For studies of the effect of radiation on exchange in other organisms, the general references at the beginning of this section may be consulted.

MODIFICATION

The experimental modification of radiation damage has become one of the most active aspects of radiation biology. It is by now thoroughly established that most, perhaps all, forms of radiation damage can be modified by appropriate treatment either before or after irradiation (9, 90, 92). The successful procedures for ultraviolet and x-rays differ so much from each other that the two kinds of radiation will be considered separately. Although some of the basic steps must differ there are some indications of similarities also.

ULTRAVIOLET

Fairly general consideration of the modification of ultraviolet damage is contained in several papers on the bacteria (114, 115, 134, 135, 190, 212) and on the protozoa (64, 65); the earlier work is reviewed in detail by Zelle & Hollaender (215). Reactivation after ultraviolet has been obtained with visible light, high temperatures, catalase and peroxidase, and various chemicals and culture ingredients. With these procedures, especially visible light, it has been established and repeatedly confirmed [e.g. (66, 192)] that some forms of ultraviolet damage are not irrevocably established for as long as several hours after irradiation.

Photoreactivation has been extensively studied and a certain amount of new information has been added since the review by Dulbecco (47), although the process is still not thoroughly understood. In protozoa, a variety of processes including division delay, death, mutation, and changes in macronuclear structure are subject to restoration by visible light (49, 65, 119); but division delay in *Blepharisma* (88) and immobilization of the cilia in *Paramecium* (27) are not. The conclusion has been discussed in a previous section that photoreactivation acts only on nuclear material. Photoreactivation has also been reported for inactivation of the flagellate, *Astasia* (171).

Giese and his co-workers (27, 28, 33, 65, 66, 67, 70, 88) have studied a number of different aspects of photoreactivation in the ciliates. Among other findings, they show that starvation considerably increases sensitivity to ultraviolet but does not change photoreactability very much. They report (66, 67) some desensitization to ultraviolet by pre-exposure to visible light, a finding also reported for *E. coli* by Weatherwax (201). Since most investigators have reported no effects of pre-exposure to visible light, this finding is not yet understood.

In bacteria, not only inactivation but inhibition of DNA synthesis, and the production of filamentous forms can be reversed by visible light (55, 113). Cohn (35) reported on the properties of ultraviolet-irradiated *E. coli* using photoreactivation and other modifying circumstances. No photoreactivation was found in one of four strains of *Azotobacter* (71) or in two species of *Streptococcus* (15). Thus, a number of kinds of ultraviolet damage can be photorestored but there are exceptions which are not yet unequivocally explained.

Following up Anderson's (4) and their own earlier work on the subject, Stein & Harm (76, 77, 187 to 191) carried out a number of investigations on heat reactivation in *E. coli*. They show that very similar reactivation by post-treatment exposure to high (44.5°C.) temperature can be obtained for bacteria inactivated by ultraviolet, by ultraviolet-treated medium, by peroxide, and spontaneously. Reactivation occurred only before the first division and was very dependent on the composition of the medium. Inactivation by light of wave lengths 334, 366, or 560 mμ showed little heat reactivation. The recovery of *E. coli* incubated in saline after irradiation is at a maximum at about 30°C. (32).

The problem of the role of peroxides in ultraviolet damage continues to receive attention (135, 212). Earlier work on restoration of ultraviolet-irradi-

ated bacteria by catalase and peroxidase has been reviewed (134, 136) and interpreted as destruction of organic peroxides formed by the radiation. It has been reported that catalase-deficient bacteria are more sensitive to ultraviolet (142, 150) and that addition of hemin to a genetically hemin-deficient strain of *E. coli* reduced its sensitivity, presumably by restoring catalase activity (150). There are, however, a number of reasons for believing that though organic peroxides may be important H_2O_2 is not, a view supported by some work with protozoa and invertebrate gametes (69) in which it was shown that the effects of H_2O_2 and of ultraviolet failed to parallel each other in a number of important respects. The same lack of parallelism is reported for ozone (68).

Reactivation after ultraviolet exposure can also be obtained in a variety of other ways, some of which have been known for some time (215). Within the last several years, work has been done with postirradiation incubation with metabolites and metabolic inhibitors (30, 52, 80, 82, 196, 197, 198, 200) in distilled water (200) or saline (32), and with substances released from irradiated cells (203). Effects of the composition (53) and pH (202) of the plating medium and of the duration of incubation before plating (34) have also been reported. There is no obvious unifying principle in this work, although a number of hypotheses have been suggested. Perhaps the best that can be done at present is to emphasize that inactivation is not an immediately irrevocable effect of irradiation but is sensitively dependent on many details of postirradiation handling. There seems to be little question that cellular metabolism as well as radiation chemical reactions are involved, but in what way is not yet clear.

Much less work has been done on modification of ultraviolet effects by treatment before or during irradiation. The work of Giese and his co-workers on the effect of starvation on division delay in the protozoa has already been mentioned. In general, starvation increases the sensitivity, but it is unlikely that this is the result of screening by the products from the food since Brandt *et al.* (28) found that a didinium which had just ingested a paramecium was even more sensitive than the controls although, after digestion of the paramecium, it was less sensitive. A number of compounds were found to protect the flagellate *Astasia*, but the action of all except one could be explained by screening (172).

In bacteria, a varity of modifying conditions have been investigated. The resistance of *Serratia marcescens* (107, 108) to inactivation and mutation increases as the water content increases. Also, photoreactivation is found only for wet-irradiated material. Changes in DNA structure are suggested as the explanation. Bruce & Maaløe (30) have studied changes in sensitivity in *Salmonella* during synchronization of division by multiple temperature shifts. Other effects of temperature before and during irradiation have been investigated by Helmke (85) and Mefferd & Campbell (139). The latter find the least death at 24°C. with more death near the extremes. They also report less effect from irradiation in hydrogen than in air. Nitriles (173), crystal violet and eosin (83, 84), carbon monoxide (140), sodium azide (16), and in-

frared (86) have been reported to increase sensitivity of bacteria; and azide reduces photoreactivation (16). A number of agents, including sodium azide and carbon monoxide, have been reported to protect *Penicillium* spores against 2750 A ultraviolet (204). Unification and generalization of these diverse observations is not yet possible.

Ionizing Radiation

The early work on modification of the effects of x-rays on microorganisms concentrated on protective treatments, especially the oxygen effect. The most extensive study is that on *E. coli* by Hollaender, Stapleton, and their co-workers [see for review (90, 93 to 96, 180, 182, 215). Briefly, this work demonstrates that oxygen removal by replacement with an inert gas, by chemical reaction, or by bacterial metabolism accounts for a large part of the protective effects found. The protective action of certain compounds, especially those with—SH groups, cannot be entirely explained in this way. Certain—SH compounds (mercaptoethanol and cysteamine) have been the subject of more recent studies (91, 95, 96) and have been found to give excellent protection.

This group of workers attributes the oxygen effect mainly to indirect action of radicals and other diffusible substances, although they recognize that other mechanisms may also play a part. The diffusible-substance hypothesis is supported by work with microorganisms at very low temperatures. A sharp break in the inactivation curve has been reported at about 0°C. for yeast (210) and bacteria (185). Some, although a rather small, oxygen effect was found below freezing (185, 211). Similar results were obtained somewhat earlier with pollen (57, 162).

Stapleton (182) has listed reasons for thinking that the indirect effect is intracellular. On the other hand, Biagini (18 to 21) has interpreted experiments with different concentrations of *E. coli* and cysteine to mean that there is an intercellular indirect effect. There is less inactivation of bacteria in concentrated than in dilute suspensions in saline, and cysteine is a less effective protector at high bacterial concentrations. Bacteria in broth show little effect of concentration and are as insensitive as those irradiated dry. The effect of bacterial metabolism on oxygen concentration has not been explicitly taken into account in this work; and it is known that it can be very important (74, 183).

The problem of intercellular indirect effects has been analyzed in some detail for *Paramecium*. Vegetative death and division delay are largely attributable to H_2O_2 formed in the medium when the paramecia are irradiated in dilute medium with few bacteria (121, 126). As expected, there is a marked oxygen effect under these conditions; but when the intercellular indirect effect is prevented by bacteria or catalase in the medium, oxygen has no influence on these vegetative effects (122). Apparently, H_2O_2 and even other substances requiring oxygen for their formation are unimportant intracellularly. The effect of air surface reported by Wichterman & Figge (207) can, in the reviewer's opinion, be accounted for by the intercellular peroxide

effect. Shepard *et al.* (175) have also reported an intercellular indirect effect for *Didinium*. They further reported no effect on division when the didinia ate heavily of irradiated paramecia, although a few didinia died.

Lethal mutations and inactivation of kappa behave entirely differently. An oxygen effect exists which is unchanged by the presence of bacteria in the medium, and H_2O_2 and the intercellular indirect effect can be ruled out as an important factor (63, 79, 121, 122, 126). An intracellular indirect effect by HO_2 is a possible explanation, but the question has been raised for *Paramecium* whether or not there may be an effect of oxygen on the direct effect (118). The same question has been raised for the inactivation of *E. coli* (2, 3) on the basis of curves of inactivation against oxygen concentration that are steeper than might have been predicted for the HO_2 hypothesis. On the other hand, there is the evidence for a diffusion process from previously cited temperature experiments with *E. coli* (185) and from sensitive volume measurements made *in vivo* (99, 157). At present, oxygen effects on both direct and intracellular indirect action must be considered possible; but intercellular indirect action is improbable except under special circumstances.

Most recent thinking has attributed the effect of oxygen tension during irradiation to some aspect of radiation chemistry, but Laser (132, 133) has reported that metabolic inhibitors can simulate oxygen removal for bacteria and yeasts and interprets this to mean an effect through cellular metabolism. Since it is well established that pre- and postirradiation modification of x-ray damage can be produced by alterations in metabolism, the demonstration of metabolic effects is not evidence that oxygen does not also act through direct reaction with the products of radiation.

Work on chemical protectors and enhancers, in addition to that of Hollaender and his co-workers, will be mentioned briefly. Protective effects of several compounds have been reported for vegetative effects on ciliates (10, 38, 120). Hematoporphyrin has been reported to sensitize *Paramecium* to x-rays (58), but a recent abstract indicates that it was really the phenol used as a preservative of the porphyrin (59). Since oxygen is required for the effect, it is possible that peroxide formation is involved. Free-radical acceptors have been reported to protect *Pseudomonas* against x-rays (164). Synkavit, which sensitizes mammals to x-rays, was without detectable effect in irradiated *E. coli* and *S. cerevisiae* (127). Malonic and maleic acids have been reported to sensitize *Saccharomyces ellipsoides* to x-rays (116).

Postirradiation modification of x-ray damage has been investigated for *E. coli* by Hollaender, Stapleton, and their co-workers (90, 93 to 96, 180, 182, 184, 186). Reactivation or recovery is obtained by incubation for 24 hrs. at lower than normal temperatures, with a maximum recovery at some intermediate temperature (e.g., 18°C.) which varies with the strain of *E. coli* (184). Inactivation increases again as the temperature is decreased still further. Two opposing processes are suggested, one causing recovery with a temperature coeficient nearly the same as that for growth, and the other, destructive at higher temperatures (94, 182). It has also been shown that certain nutrilites promote recovery and are necessary for low-temperature

recovery and for the protective action of cysteamine (91, 94, 186). Postirradiation nutrition is less important for cells grown on minimal medium instead of broth before irradiation. A possible interpretation of these results is discussed in the section on inactivation.

Pratt *et al.* (160) report a postirradiation temperature effect for *E. coli* that seemed to them to be different. The irradiated bacteria were held at 4° to 5°C. and plated at various intervals. There were considerably more survivors in platings made after 6 days in the cold than in those made immediately. No nutritive requirement for this recovery was found. Since Stapleton *et al.* (184) report a considerable difference between strains, it is possible that the strain used by Pratt *et al.* has a low-temperature optimum.

An effect of postirradiation temperature on recovery from division delay in *P. aurelia* has been reported after ultraviolet (125) and x-rays (158). The two investigations are not easily compared because of differences in procedure and interpretation. Powers (158) suggests that the action of temperature is opposite for the two radiations, but the reviewer believes they are the same. In both cases, there is a more rapid recovery at low temperature when the results are considered in terms of the number of divisions instead of time after irradiation.

At the other extreme of temperature, Langendorff & Sommermeyer (130, 131) have reported heat (45°C.) reactivation of *E. coli* treated with x-rays or polonium alpha particles. They have also reported that preirradiation incubation at 37° instead of 20°C. sensitized the bacteria to x-rays but not to alpha particles.

Beutler *et al.* (17) found no effect of various substances and tissue extracts on x-ray-induced prolongation of the lag period in *E. coli.* Wainwright & Nevill (199) found that postirradiation incubation of irradiated *Streptomyces* spores in distilled water increased survival. The increase was prevented if casein hydrolyzates or NH_4Cl was added, but iodoacetate decreased survival. The results are different in several respects from those obtained with ultraviolet (197, 198, 200). Ducoff (45) has presented evidence for the ciliate *Tetrahymena* to indicate that some recovery from x-ray-induced lag in division occurs in the absence of exogenous amino acids provided purines and pyrimidines are supplied.

SUMMARY

A number of effects of radiation which are not immediately and obviously genetic have been detected by physiological and biochemical methods. Many of the effects detected at low doses can probably be interpreted best as inhibitions of the synthesis of specific macromolecular constituents; and there is direct evidence for inhibition of the synthesis of DNA and certain adaptive enzymes. Nevertheless, there is as yet only a small amount of information on the relation of these observed effects to the primary lesions or to one another. There are a number of reasons for thinking that the effective primary lesions at low doses are mainly nuclear, and at least part of this nuclear damage must be readily reversible. There is, however, also some

reason for believing that cytoplasmic damage may be of some importance, at least in those organisms which are able to avoid the effects of low doses.

A very considerable amount of work has been done on the modification of radiation damage. Certain general types of modifying procedures are by now well established, and reasonable theories to account for some of the effects are available. The demonstration that damage by ionizing as well as ultraviolet radiation is modifiable after exposure shows that there is an appreciable interval between absorption of the radiation and the final establishment of at least part of the damage.

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NUTRITION OF BACTERIA AND FUNGI[1,2]

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This review of microbial nutrition may appropriately open with a tribute to the late J. Howard Mueller, Professor of Bacteriology and Immunology at Harvard University, whose death on February 16, 1954, ended a career of outstanding achievement in the field of bacterial nutrition. Mueller's first publication on the cultural requirements of bacteria (1) in 1922 stated his belief that the "empirical mixtures containing infusions of meat, the digestion products of protein, and so forth" owed their ability to support the growth of pathogenic bacteria to certain basic growth factors contained in them; the successful isolation and identification of these growth factors by chemical manipulation might have application to

> problems of more general biological importance, particularly to those of animal metabolism. For whatever may prove to be the nature of these substances which cause growth of bacteria, they are largely or entirely components of animal tissue, and it is probable that they are needed also by the animal body and supplied by plant or other sources, or else are synthesized by the animal itself to fill some metabolic requirement. When it is possible to catalogue the substances required by pathogenic bacteria for growth, it will probably be found that most of them are either required by, or important in, animal metabolism, and while many of them will surely be compounds at present familiar to the physiological chemist, it is equally probable that some will be new or at least of hitherto unrecognized importance.

The soundness of Mueller's approach is illustrated by his discovery in 1923 of a new amino acid, methionine, by the fractionation of the hydrolytic products of casein which are required for the growth of streptococci (2). This was the first instance of the discovery of a new compound of general biological importance through the study of bacterial nutrition.

The rapid advance made in the study of the growth requirements of many fastidious microorganisms in the last 25 years owes a great deal to the adoption of Mueller's analytical approach. His own contributions include the development of media of completely known composition capable of supporting full growth of the diphtheria bacillus (3) and of the tetanus bacillus (4). During the last years of his life, he had turned his attention to the requirements for toxin production by *Clostridium tetani*. He was able to show

[1] The survey of literature pertaining to this review was completed in January, 1957.

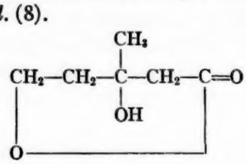
[2] The following abbreviations are used: ADP (adenosine diphosphate); ATP (adenosine triphosphate); DNA (deoxyribonucleic acid); CTP (cytidine triphosphate); GDP (guanosine diphosphate); GTP (guanosine triphosphate); PABA (*p*-aminobenzoic acid); RNA (ribonucleic acid); and UTP (uridine triphosphate).

that peptides, though not required for growth, were necessary for the production of the toxin (5).

Progress in microbial nutrition has been reviewed at yearly intervals in this publication, and the reader can readily see that the study of the growth requirements of bacteria, true to Mueller's prediction of 1922, has contributed greatly to our understanding of problems of general biological importance. Indeed, the achievements in the study of the cultivation of microorganisms have been so great that it seems doubtful that many growth factors of outstanding importance remain to be discovered. However, in other respects the study of bacterial nutrition continues to provide results of general biological importance. In particular, the study of the nutrition of microbial auxotrophic mutants in combination with the study of their enzymatic composition is an invaluable tool for the elucidation of the biosynthetic pathways leading to the essential components of the cell. More recently, the profound effect of the nutrient medium on the enzymatic constitution and the metabolic activities of microbial cells has been recognized; it would appear that the constituents of the growth medium regulate the synthesis of the microbial protoplasm by controlling the entry of nutrients into the cell as well as their subsequent intracellular metabolism. The elucidation of these phenomena may contribute to the understanding of the coordination of the many individual enzymatic reactions occurring in the same cell, which is a necessary attribute of life. The present review is largely devoted to a discussion of these aspects of microbial nutrition.

Nutrition and Biosynthesis

Fatty acids and lipides.—The chemistry and metabolism of the lipides has remained one of the least explored areas of biology. However, some progress has been made during the past year in the elucidation of the role of certain fatty acids and other constituents of lipides in microbial nutrition. An important contribution is the discovery of a compound capable of replacing acetate in the nutrition of *Lactobacillus acidophilus* and of certain other lactobacilli by Skeggs *et al.* (6), its isolation in pure form from dried distillers' solubles by Wright *et al.* (7), and its identification by structural degradation and by synthesis as β-hydroxy-β-methyl-δ-valerolactone (divalonic acid) by Wolf *et al.* (8).



The compound supports full growth of *L. acidophilus* in the test system at a concentration of approximately 0.02 μg. per ml. and cannot be replaced by lipoic acid which is without effect on the growth of the microorganism. The small amount of divalonic acid required (it is about 20,000 times as ac-

tive as acetate) places it in the category of microbial vitamins; its biological role is not known but it has been shown by Tavormina, Gibbs & Huff (9) to be an excellent precursor of cholesterol in rat liver homogenates. This is in good accord with early observations by Guirard *et al.* (10) that steroids and the nonsaponifiable lipide fraction of bacteria were several hundred times as active as acetate in supporting the growth of *L. acidophilus.*

A surprising finding is the observation of Shockman (11) that three strains of *Streptococcus faecalis* which require acetate, or lipoic acid and thiamine, for aerobic growth can grow without these substances under anaerobic conditions; cells grown aerobically on acetate or anaerobically do not contain lipoic acid.

Camien & Dunn (12, 13, 14) have continued their studies of compounds capable of replacing D-lactic acid in the nutrition of a mutant of *Lactobacillus casei* which has lost the ability to convert L-lactic acid to D-lactic acid. They found that the organism can grow on D-α-hydroxy acids having a chain length of 3 to 14 carbon atoms as well as on D-1,2-alkanediols with a chain length of 12 or 14 carbon atoms; D-α-hydroxycaproic acid and D-α-hydroxycaprylic acid had the highest growth-supporting activity. The authors suggest D-lactic acid to be the normal precursor of the cerebronic acids of the organism, which by analogy with those of brain, yeast, and wool wax cerebrosides may be considered to consist of α-hydroxy acids of the D series.

The role of *myo*-inositol, a constituent of phospholipides, in the nutrition of certain yeasts and molds was investigated in several laboratories. Posternak & Schopfer (15) found that the *myo*-inositol antagonist, *iso*-mytilitol, which differs from *myo*-inositol by carrying a methyl group in place of the hydrogen on carbon 2, was incorporated into the lipides of an inositol-less strain of *Neurospora crassa*, apparently replacing *myo*-inositol in phosphatides which normally constitute 8 per cent of the lipides of this organism. The effect of *iso*-mytilitol thus appears to be analogous to that of azaguanine,5-bromo-uracil, and certain structural analogues of amino acids, which all act by being incorporated into a cell constituent in place of the corresponding normal metabolite. *myo*-Inositol deficiency is indicated to be the cause of a disturbance of lipide metabolism by the report of Chalinor & Daniels (16) that inositol-requiring yeast cells grown in an inositol-deficient medium were easily ruptured and contained 24.5 per cent lipide which is present to the extent of only 1.6 per cent in normal yeast cells. An entirely different metabolic role for *myo*-inositol is suggested by the observation of de Robichon-Szulmajster (17) that certain pyrimidineless mutants of *Saccharomyces cerevisiae* can grow in the absence of *myo*-inositol when supplied with cytosine, uridine, or uridylic acid, but require *myo*-inositol for growth with uracil as the source of pyrimidine; another mutant of this organism requires inositol irrespective of the pyrimidine supplied, whereas a third mutant requires *myo*-inositol but no pyrimidine. In all cases 2-keto-*myo*-inositol and D,L-1-keto-*myo*-inositol can substitute for *myo*-inositol. It is difficult to fit these observations into the current concepts of pyrimidine biosynthesis.

Amino acids.—Microbial nutrition has played a key role in the rapid progress of the study of amino acid metabolism during the last decade. A recent authoritative review of amino acid biosynthesis by Davis (18) presents a clear picture of the role of amino acids and of their precursors in the growth of microorganisms.

During the past year the metabolic relations between L- and D-threonine, α-ketobutyric acid, α-aminobutyric acid, and L-isoleucine, and the role of these compounds in the nutrition of mutants of *Escherichia coli* have been clarified by Umbarger (19, 20, 21). Earlier work (22) had shown that in addition to mutants with an absolute requirement for L-threonine (class 6), others existed which required L-isoleucine, D-threonine, α-ketobutyric acid, or α-aminobutyric acid, but could not grow on L-threonine (class 4). It has now been shown that the mutants of class 4 grown in a minimal medium containing the growth factor and glucose, lack an enzyme, L-threonine dehydrase, which converts L-threonine to α-ketobutyric acid and which is present in extracts of the wild strain and of mutants of class 6. This enzyme appears therefore to be essential for the formation of L-isoleucine, and, consequently, L-threonine and α-ketobutyric acid must be considered intermediates in the biosynthesis of L-isoleucine from glucose. The growth supporting activity of α-amino-butyric acid and of D-threonine for mutants of class 4 may be explained by the presence of enzymes, α-aminobutyrate-pyruvate transaminase (23) and D-threonine dehydrase (19), capable of converting these compounds to α-ketobutyric acid. The mutants of class 6 appear to be unable to synthesize L-threonine which has to be supplied and serves as the precursor of both the L-threonine and L-isoleucine of the bacterial protein as shown by experiments with C^{14}-labelled L-threonine (18). Isoleucine spares the requirement of mutants of class 4 for L-threonine by inhibiting the enzymatic conversion of L-threonine to α-ketobutyric acid; the significance of this inhibition as a control mechanism will be discussed in a later section of this review.

The studies of Davis, Sprinson and their co-workers (24 to 27) have clarified the early steps in the biosynthesis of the aromatic amino acids from glucose and demonstrated the condensation of D-erythrose-4-phosphate with phosphoenolpyruvate to 5-dehydroquinic acid in bacterial extracts. Studies on the formation of protocatechuic acid indicate a similar pathway in *Neurospora* [Tatum & Gross (28)]. The enzymatic steps by which 5-dehydroquinic acid is converted to shikimic acid had been previously elucidated by Davis and his co-workers [see (18)]. Shikimic acid is apparently converted to 5-phosphoshikimic acid which has been isolated from the culture fluids of bacterial mutants blocked in the biosynthesis of aromatic compounds and identified by Weiss & Mingioli (29). The enzymatic condensation of this phosphate ester with phosphoenolpyruvate to Z1, an as yet unidentified compound accumulating in the culture fluids of aromatic auxotrophs blocked after shikimic acid (18), has been observed by Kalan (30); compound Z1

yields shikimic acid and pyruvic acid on acid hydrolysis [Gilvarg (31)], and may be a common precursor of aromatic metabolites of microorganisms. Zl is structurally related to prephenic acid which has previously been shown to be a precursor of phenylpyruvic acid and phenylalanine (18), and is now reported to yield *p*-hydroxyphenyllactic acid when incubated with extracts of a mutant of *E. coli* blocked between prephenic acid and phenylpyruvic acid [Ghosh *et al.* (32)]. *p*-Hydroxyphenyllactic acid may be converted to tyrosine, presumably via *p*-hydroxyphenylpyruvic acid.

A study by Holden (33) on the effects of phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid on the growth of *S. faecalis* and of *Lactobacillus arabinosus* supports the hypothesis that these ketoacids are the precursors of the corresponding amino acids, phenylalanine, and tyrosine. In tyrosine-deficient media, vitamin B_6 was required for growth and phenylpyruvic acid was inhibitory; similarly, in phenylalanine-deficient media, vitamin B_6 was required and *p*-hydroxyphenylpyruvic acid was inhibitory. The results are explained by the assumption that the amino acids arise from the ketoacids by pyridoxalphosphate-linked transaminations, and that the exogenous ketoacid competes with the endogenously formed ketoacid for the transaminase.

The conversion of anthranilic acid to indole has been studied by Yanofsky (34). Extracts of a trytrophanless mutant of *E. coli* which excretes indole, catalyze the condensation of anthranilic acid with 5-phosphoribosyl pyrophosphate to indole-3-glycerolphosphate and the cleavage of this compound to indole and triosephosphate. These results, which are in agreement with the results of isotopic and nutritional studies (18, 35, 36, 37), clarify the role of anthranilic acid and of indole in the biosynthesis of tryptophan, and explain the ability of these compounds to replace tryptophan in the nutrition of certain bacteria [see (18)].

The detailed discussion of histidine biosynthesis in last year's review (38) included the consideration of Hartman's studies on *Salmonella typhimurium* now published in full (39).

The requirement of mutants of *E. coli* and *Aerobacter aerogenes* for glutamic acid or α-ketoglutaric acid was traced by Gilvarg & Davis (40) to the loss of the citrate-condensing enzyme. The ability of histidine and of citric acid to replace glutamic acid in the nutrition of the *A. aerogenes* mutant will be discussed later in this review.

Barratt *et al.* (41) have studied a mutant of *N. crassa* with an alternative requirement for leucine and phenylalanine, and found it to be capable of synthesizing both amino acids *de novo* and to be incapable of converting leucine to phenylalanine or phenylalanine to leucine; the nature of the genetic block responsible for the growth requirement remains obscure.

The interactions between carbohydrate and nitrogen metabolism in a succinate requiring mutant of *N. crassa* were examined by Strauss (42).

Two peptides possessing streptogenin activity for *L. casei* were isolated

by Merrifield & Woolley (43) from acid-hydrolyzed crystalline beef insulin and their structures determined; one of them, the pentapeptide Ser. His. Leu. Val. Glu., was synthesized (44).

Appleyard, Nimmo-Smith & Woods (45, 46) have studied a pseudomonad able to use creatine as the sole source of carbon and nitrogen.

Purines.—The biosynthesis of purines and their role in microbial nutrition have been considerably clarified in recent years. The study of purine requiring mutants of *E. coli*, *S. typhimurium* and *A. aerogenes* by Gots and his co-workers (47 to 51) and by Magasanik & Brooke (52, 53) has shown that these mutants may be divided into four classes according to their response to the common naturally occurring purines: hypoxanthine, xanthine, guanine, and adenine. Class 1 comprises those mutants which can grow on any one of these four compounds; class 2 consists of mutants which can grow on xanthine or guanine, but not on hypoxanthine or adenine; class 3 comprises the mutants with a specific requirement for guanine, and class 4 those with a specific requirement for adenine.

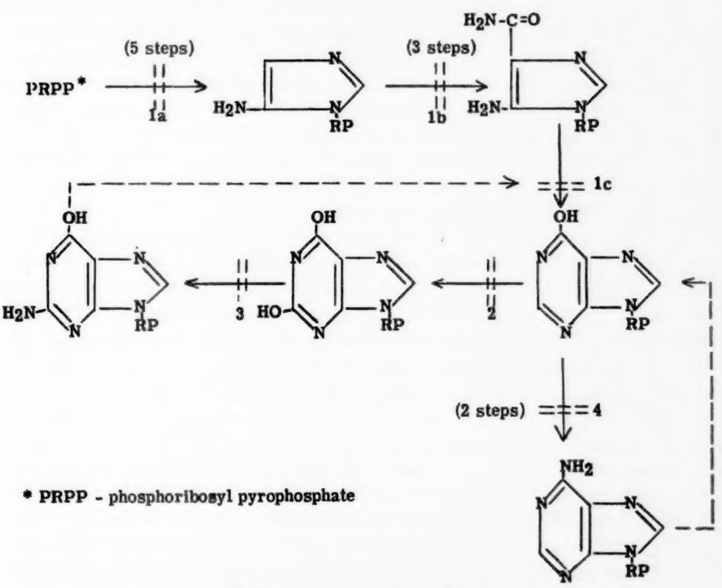
Mutants of class 1 seem to lack enzymes essential for the biosynthesis of inosine-5′-phosphate as indicated by their excretion or utilization of imidazole derivatives which correspond to the imidazole ribotides identified as intermediates in the biosynthesis of inosine-5′-phosphate in avian liver by Buchanan and his co-workers and by Greenberg and his co-workers [reviewed by Carter (54)]. Class 1 may be further subdivided according to the excretion of these diazotizable imidazole amines: strains belonging to class 1a do not excrete any diazotizable amines, whereas strains belonging to classes 1b and 1c excrete the ribosides of 4-amino-imidazole and of 4-amino-5-imidazolecarboxamide, respectively; 4-amino-5-imidazolecarboxamide can replace purine in the nutrition of the mutants of classes 1a and 1b.

The conversion of inosine-5′-phosphate to a derivative of guanine requires two steps. Inosine-5′-phosphate is first oxidized to xanthosine-5′-phosphate with diphosphopyridine nucleotide serving as the hydrogen acceptor; mutants of class 2 lack the required enzyme, which therefore appears to be essential for the synthesis of guanine nucleotides *de novo*, or from hypoxanthine or adenine [Gehring & Magasanik (55); Magasanik, Moyed & Gehring (56)]. Xanthosine-5′-phosphate is converted to guanosine-5′-phosphate through amination by ammonia with the concomitant cleavage of ATP to adenosine-5′-phosphate and pyrophosphate; the lack of the enzyme which catalyzes this reaction is responsible for the specific guanine requirement of mutants of class 3 [Moyed & Magasanik (57)].

The conversion of inosine-5′-phosphate to an adenine derivative also occurs in two steps. Lieberman (58) has found an enzyme in extracts of *E. coli* which catalyzes the condensation of inosine-5′-phosphate and aspartic acid to adenylosuccinate with the concomitant cleavage of GTP to GDP and inorganic phosphate. Adenylosuccinate is hydrolyzed by another enzyme in this extract to adenosine-5′-phosphate and, presumably, fumarate.

Adenine requiring mutants of *N. crassa* have been shown (59, 60) to fall into two groups according to their excretion or lack of excretion of succinyladenine; the mutants excreting this compound lack the enzyme necessary for the conversion of adenylsuccinate to adenosine-5′-phosphate. On the basis of these results, the specific requirement of mutants of class 4 for adenine may be ascribed to the lack of the adenylosuccinate-condensing enzyme or to the lack of adenylosuccinase. The conversions of inosine-5′-phosphate to adenosine-5′-phosphate and to guanosine-5′-phosphate in animal tissues involve similar reactions (see 54).

The pathway of purine biosynthesis and the positions of the genetic blocks is summarized in the following diagram:



Pathway of purine biosynthesis

The entry of the exogenous purines or purine ribosides into this pathway requires their conversion to nucleoside-5′-phosphates, presumably by reaction with 5-phosphoribosyl pyrophosphate or ATP, respectively. In order to support the growth of mutants belonging to class 1, the purine fed must be converted to inosine-5′-phosphate. In the case of adenine, this may involve the reversal of the enzymatic reactions leading from inosine-5′-phosphate to adenosine-5′-phosphate; however, in the cases of guanine and xanthine, the irreversibility of the enzymatic reactions leading from inosine-5′-phosphate to guanosine-5′-phosphate indicates that the conversion must occur by

means other than the reversal of these steps (56, 57). Existence of such a pathway is supported by the observation that a mutant of class 3 which lacks xanthosine-5′-phosphate aminase is able to convert guanine but not xanthine to nucleic acid adenine, perhaps by the replacement of carbon 2 of guanine by a single carbon unit [Balis *et al.* (61); Magasanik *et al.* (62)]. Isoguanine can replace xanthine, to which it is converted by a deaminase found in *E. coli* by Friedman & Gots (63). 2,6-Diaminopurine can replace guanine in the nutrition of mutants of class 3 which are capable of synthesizing adenine *de novo*, but does not support the growth of mutants of class 1, presumably because of the sluggishness of its conversion to adenine derivatives (53, 61).

The transfer of carbon atom 2 of guanine to an unknown precursor of histidine to become carbon 2 of the imidazole ring of histidine was first observed in *L. casei* by Mitoma & Snell (64), and later in *A. aerogenes* and *E. coli* by Magasanik *et al.* (62). These results are in accord with the sparing by histidine of the purine required for the growth of *L. casei* and of a mutant of *E. coli* (62, 65). In this mutant, guanine was found to serve as the sole source of the N_1-C_2 portion of the imidazole ring of histidine; the remainder of the guanine molecule was apparently excreted as the riboside of 4-amino-5-imidazolecarboxamide [Magasanik (66)].

A further demonstration of the metabolic relationship of histidine and purines is the ability of histidine to overcome the inhibitory action of adenine on the growth from small inocula of wild strains, as well as purineless mutants of *A. aerogenes*, *E. coli*, and *S. typhimurium* [Brooke (67)]. This inhibition can be reversed also by succinic acid and other components of the citric acid cycle, by pantothenic acid, and by thiamine, but not by guanine (50, 67). Mutants of class 1a, however, seem to require thiamine which cannot be replaced by histidine, succinic acid, or pantothenic acid, even when guanine or xanthine is the source of purine (53, 67). The role of thiamine in the metabolism of purines, or conversely, the role of purines in the metabolism of thiamine, remains to be clarified.

The scheme of purine biosynthesis and interconversions discussed here would appear to account in a satisfactory manner for the marked differences found in the utilization of purines by a variety of yeasts, molds, bacteria, and protozoa discussed by Brown (68). The observation that a yeast *Torulopsis utilis*, which does not require purines incorporates exogenous adenine into both nucleic acid adenine and guanine, but incorporates exogenous guanine only into nucleic acid guanine (69, 70), may be explained by the inability of this organism to convert guanine to inosine-5′-phospate (see diagram). Similarly, the wild strain of *N. crassa* seems to lack the mechanism for the conversion of guanine to inosine-5′-phosphate, for mutants of this organism requiring adenine or hypoxanthine, but unable to grow on xanthine or guanine have been described (71); these mutants presumably have lost the ability to synthesize inosine-5′-phosphate *de novo*.

Certain species of bacteria such as *L. casei*, though unable to grow with-

out exogenous purine in media free of folic acid, can utilize any one of the common purine bases and in this respect resemble the mutants of class 1 described before (72, 73).

Micrococcus pyogenes var. *aureus* does not require purines for growth and can derive both nucleic acid purines from exogenous guanine, but only nucleic acid adenine from exogenous adenine (74); a mutant of this organism selected by its resistance to chloramphenicol requires adenine together with either hypoxanthine or xanthine or guanine, and is not able to derive its nucleic acid adenine from exogenous guanine. In this case the parent strain may lack the ability to convert adenine to inosine-5′-phosphate and the mutant may have lost in addition the capacity for the synthesis of inosine-5′-phosphate *de novo* and for its conversion to adenosine-5′-phosphate.

Corynebacterium diphtheriae requires hypoxanthine or a mixture of adenine and guanine for growth (75); this organism would seem to lack the ability to synthesize inosine-5′-phosphate *de novo* or from adenine or guanine.

Tetrahymena gelei requires guanine, which serves as the sole source of both nucleic acid purines (76, 77, 78); adenine, though incapable of supporting its growth is incorporated into nucleic acid adenine, but not into nucleic acid guanine; it would appear that this organism cannot synthesize inosine-5′-phosphate *de novo* and cannot convert it to guanosine-5′-phosphate. A similar pattern of purine utilization is also found in *Lactobacillus leichmanii* (79) and in certain mutants of *Ophiostoma* (80).

Pyrimidines.—A recent paper by Yates & Pardee (81) confirms that the pathway of pyrimidine synthesis in *E. coli* involves the conversion of aspartate and carbamyl phosphate to uridine-5′-phosphate by the enzymes discovered by Jones *et al.*, and by Kornberg and his co-workers (see 54).

$$\text{Aspartate} + \text{Carbamyl phosphate} \rightarrow \text{Ureidosuccinate} \rightleftarrows \text{Dihydro-orotate} \rightleftarrows \text{Orotate}$$

$$\xrightleftharpoons{\text{PRPP}} \text{Orotidine-5}'\text{-phosphate} \rightarrow \text{Uridine-5}'\text{-phosphate} \rightarrow \text{UTP} \rightarrow \text{CTP}$$

This pathway of pyrimidine biosynthesis accounts for the role of orotic acid which can replace uracil in the nutrition of many organisms (82) and explains the existence of mutants with a requirement for both a pyrimidine and arginine or citrulline, which apparently are unable to form carbamyl phosphate (18, 83).

Uridine-5′-phosphate appears to be the precursor of nucleic acid uracil, cytosine, and thymine. The conversion of uridine-5′-phosphate to a cytosine derivative probably occurs by means of an enzyme discovered by Lieberman (84) in extracts of *E. coli* which catalyzes the amination of UTP by ammonia in the presence of ATP which is concomitantly cleaved into adenosine diphosphate and inorganic phosphate. So far, no mutant with a specific requirement for cytosine, indicating the lack of this enzyme, has been described. On the other hand, most pyrimidineless mutants can grow on either uracil or cytosine or the corresponding nucleosides; this may reflect the wide distribution of cytosine deaminase which can convert cytosine to uracil (85).

The conversion of exogenous uracil-C^{14} to nucleic acid thymine has been demonstrated (86); recently Friedkin & Kornberg (87) have observed in bacterial extracts an enzymatic reaction of deoxyuridylic acid with hydroxymethyltetrahydrofolic acid to give a thymine nucleotide. The suggestion that dihydropyrimidine nucleotides may be intermediates in this reaction has not yet been experimentally verified (88). A mutant of *E. coli* with a requirement for dihydrouracil has been described (89); however, dihydrouracil cannot be replaced by thymine or thymidine and therefore cannot without additional evidence be considered a precursor of these compounds. Dihydrothymine can replace thymine in the nutrition of *S. faecalis* though the latter is about 1500 times as active (90). Thymidine can be replaced in the nutrition of *L. leichmanii* and of *L. acidophilus* R26 by the deoxyriboside of 5-bromouracil (91); in contrast to thymidine this compound is only active in the presence of folic acid and presumably supplies the deoxyribose required by these organisms.

Vitamins.—The relationship between *p*-aminobenzoic acid and folic acid derivatives in *E. coli* has been investigated by Davison *et al.* (92) who fed radioactive PABA to a PABA-requiring mutant of *E. coli* and isolated from cell extracts by chromatographic methods four labile radioactive components which were not identical with folic acid or citrovorum factor. Two of the radioactive fractions could replace folic acid and citrovorum factor, respectively, in the nutrition of *S. lactis* R. and of *L. citrovorum*, but all were unable to support the growth of the PABA-requiring mutant from which they had been isolated. This report confirms the inability of *E. coli* and related organisms to utilize folic acid derivatives in place of *p*-aminobenzoic acid. Whether this failure is due to a permeability barrier or to the lack of enzymes capable of transforming the folic acid derivatives to the metabolically active forms of folic acid remains unknown. Recent experiments by Koft & Morrison (93) have shown that *Acetobacter suboxydans* requiring PABA and unresponsive to folic acid, and *S. faecalis*, strain R, requiring folic acid and unresponsive to PABA, can grow symbiotically in a culture devoid of both nutrilites. These results suggest that each organism excretes compounds which can be used by the other and may offer an approach to the identification of as yet unknown intermediates between PABA and the active form of folic acid.

The ability of triglutamylpteridines corresponding to citrovorum factor and to N-10-formylfolic acid to replace citrovorum factor or folic acid in the nutrition of bacteria was investigated by Silverman & Wright (94). The compounds isolated from extracts of *Clostridium cylindrosporum*, which are cofactors in the conversion of serine to glycine by a bacterial enzyme system, could replace folic acid and citrovorum factor in the nutrition of *L. casei*, but not of *S. faecalis* R. and of *L. citrovorum;* treatment with rat liver extract converted them to compounds possessing the expected activity for these organisms.

Other derivatives of folic acid which have recently been shown to arise in

reactions catalyzed by bacterial extracts and to be capable of acting as coenzymes in enzymatic reactions are N-5-formiminotetrahydrofolic acid, formed by the transfer of a formimino-group from formiminoglycine to tetrahydrofolic acid, and N-10-formyltetrahydrofolic acid, formed with the loss of ammonia via the N-5-N-10-imidazolinium derivative of tetrahydrofolic acid from N-5-formiminotetrahydrofolic acid. N-10-formyltetrahydrofolic acid in the presence of ADP and inorganic phosphate is enzymatically cleaved to formic acid and tetrahydrofolic acid with the concomitant production of an equimolar amount of adenosine triphosphate [Rabinowitz & Pricer (95 to 97)]. It is not known whether these derivatives of tetrahydrofolic acid are able to replace PABA or folic acid derivatives in the nutrition of microorganisms.

The isolation from human urine of the pteridine required for the growth of *Crithidia fasciculata*, its characterization and synthesis have been reported by Patterson *et al.* (98, 99): the compound, biopterin, was identified as 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine. Nathan *et al.* (100) report that *C. fasciculata* possesses independent requirements for folic acid and biopterin. The organism requires either a high concentration of folic acid (300 mμg./ml.) or a mixture of biopterin (0.1–1.0 mμg./ml.) and of folic acid (1–3 mμg./ml.), or a mixture of biopterin (0.1–1 mμg./ml.) and of thymine (10,000 to 30,000 mμg./ml.). Biopterin, in the absence of either folic acid or thymine, is not capable of supporting the growth of this organism. The authors deduce from these experiments that folic acid, when it is the sole source of pteridines, functions in 1-carbon metabolism and is, in addition, the precursor of a class of pteridines which has an independent function. Biopterin appears to be superior to folic acid as a precursor of this class of pteridines; the sparing effect of biopterin on the requirement of the protozoon for riboflavin is interpreted as indicating a biosynthetic relationship between folic acid, biopterin, and riboflavin.

Recent observations suggesting for biotin a role in purine biosynthesis accord well with the frequently demonstrated relationship between biotin and the metabolism of aspartic acid and of carbon dioxide. The excretion of a derivative of 4-amino-imidazole by a biotin-requiring yeast originally observed by Chamberlain *et al.* (101) was further investigated by Moat *et al.* (102) who found that it could be prevented by the addition of an amount of biotin optimal for growth, or by the addition of purines, to the culture fluid. It would seem that the biotin-deficient yeast cannot carry out the reaction of 4-aminoimidazole ribotide with aspartic acid and carbon dioxide which yields 4-amino-5-imidazole carboxamide ribotide (103). Estes, Ravel & Shive (104) report that extracts of biotin-deficient *Streptococcus lactis* have considerably less ability to convert ornithine to citrulline than extracts of cells grown on a medium rich in biotin; these results are interpreted as indicating that a biotin derivative functions as a cofactor in the carbamylation of ornithine. However, as it has not been possible to reactivate the extracts of deficient cells by the addition of biotin, the evidence

for this role of biotin cannot as yet be considered conclusive. The excretion of nicotinic acid by biotin-requiring yeasts was prevented by biotin [Rose & Nickerson (105)]; this biotin-nicotinic acid relationship could conceivably be a result of the reduced capacity of biotin-deficient cells to produce the purine moiety of the pyridine containing coenzymes.

Vitamin B_{12}, according to the work of Downing & Schweigert (106), is involved in the biosynthesis of the deoxyribose moiety of deoxyribonucleotides. These investigators found that *L. leichmanii* grown in a medium containing uniformly C^{14}-labelled thymidine but no B_{12} derived its deoxyribose almost exclusively from the proffered thymidine; in the presence of vitamin B_{12} the bulk of the nucleic acid deoxyribose was derived by synthesis from unlabelled precursors. Earlier work by Rose & Schweigert (107), as well as a recent study by Roll *et al.* (108), indicate the ribosides or ribotides to be the direct precursors of the deoxyribosides or deoxyribotides in mammalian tissues, and vitamin B_{12} may be involved in these reactions. The role of vitamin B_{12} in deoxyribotide synthesis is apparently not in accord with the observation that methionine, in the absence of deoxyribosides, is capable of replacing vitamin B_{12} in the nutrition of a mutant of *E. coli* (109); the synthesis of methionine from homocysteine by cell-free extracts of this mutant is stimulated by the addition of cyanocobalamine (110). Dubnoff & Barton (111) report that the activities of many enzymes of a mutant of this type measured in resting cells are stimulated by the addition of vitamin B_{12} and glutathione; they suggest that vitamin B_{12} may be involved in maintaining the activities of essential sulfhydryl-containing enzymes. The growth supporting activities of several pseudovitamin B_{12} compounds for five different organisms were determined by Peterson *et al.* (112).

Miscellany.—The aerobic cultivation of *C. tetani* in the presence of cobalt was claimed by Dedic & Koch (113) and refuted by Lev (114). The ability of a mutant of *E. coli* to use nitrate as the sole source of nitrogen was examined by McNall & Atkinson (115); other studies have dealt with the nutrition of marine bacteria (116), of urea-hydrolyzing bacteria (117, 118), of some species of the genus *Bacillus* (119), of *Bacterium salmonicida* (120), of putrefactive anaerobic bacteria (121), of *Actinomyces* (122), of *Myxococcus virescens* (123) and of the wheat bunt fungus (124). The stimulation of bacterial growth by substances from corn steep liquor (125), from yeast and liver extracts (126), and of heat degradation products of glucose (127) has been observed.

Nutrition and Membrane Transport

Transport of amino acids.—Earlier studies by Gale (128) and by Halvorson & Spiegelman (129) had revealed the energy-requiring, intracellular concentration of amino acids in staphylococci and in yeast. Cohen & Rickenberg (130) have now discovered a similar intracellular concentration of valine and other amino acids in *E. coli*. The uptake of valine was measured by exposing the cells to radioactive valine under aerobic conditions, collect-

ing the cells by centrifugation, and measuring the amount of radioactivity that could be extracted by boiling water. In this simple manner it could be shown that the concentration of valine occurred rapidly, a maximum being reached in about 1 min. at 37°C. and in 20 min. at 0°C.; that it required an energy source, succinate or glucose, and was prevented by inhibitors acting on energy metabolism such as 2,4-dinitrophenol and azide; that it was proportional to the number of bacteria and that its dependence on the exogenous concentration of valine followed a classical adsorption isotherm, reaching a maximum (about 3.6×10^6 molecules of valine per cell, corresponding roughly to one hundred fold concentration) at an exogenous L-valine level of $2 \times 10^{-5}M$. The uptake of valine was faster than its incorporation into bacterial protein and occurred also under conditions that did not permit the synthesis of protein, for instance in a methionineless mutant deprived of methionine, and in the wild strain in the presence of chloramphenicol. An important feature of the phenomenon of intracellular valine concentration in *E. coli* is its reversibility. Unlabelled L-valine, L-isoleucine, L-leucine and, to a lesser extent, nor-leucine prevented the uptake of valine-C^{14} by the cells and, if added after the uptake of valine-C^{14} had taken place, brought about its removal from the cell. The corresponding amino acids of the D-series, amides, and peptides, and L-amino acids structurally unrelated to valine, did not affect the uptake of labelled valine. Similar concentration mechanisms for L-phenylalanine and for L-methionine were also demonstrated, but not studied in detail. The uptake of proline by *E. coli* was studied by Britten *et al.* (131) and found to occur through a similar mechanism. The existence of these transport mechanisms for amino acids in *E. coli* is in agreement with the observation by Mandelstam (132), that Gram-negative organisms contain an intracellular pool of amino acids, which, however, is smaller than that of Gram-positive organisms.

An important contribution to the knowledge of bacterial nutrition is the demonstration in these studies that structurally related amino acids can specifically interfere with the uptake of one another by the cell. This antagonism, ascribed by Cohen & Rickenberg to competition for a sterically specific "permease" catalyzing the transport of these amino acids across the cell membrane, agrees with the explanations advanced by other workers to account for the growth-inhibitory activity of certain amino acids. Thus, Umbarger & Brown (133) observed that isoleucine inhibited the growth of mutants of *E. coli* requiring exogenous valine, but did not affect the growth of the wild strain; the inhibitory action of isoleucine was overcome competitively by valine, but noncompetitively by α-keto-isovalerate, the keto analogue of valine, and by glycylvaline. Earlier, Prescott *et al.* (134) had shown that the inhibitory action of L-alanine on the growth of *Lactobacillus delbrueckii* could be overcome by any one of a series of L-serine-containing peptides. In both cases, the results were interpreted as indicating a competition between isoleucine and valine, and between alanine and serine, respectively, for transport across the cell membrane. It was postulated that

the passage of the ketoacid and of the peptides did not involve the specific sites of the cell membrane which were responsible for the transport of the amino acids, and was consequently not prevented by isoleucine or alanine. The interesting finding that α-keto-β-methyl-valerate, the keto analogue of isoleucine, interferes with the growth of the *E. coli* mutant on α-ketoisovalerate, but not with its growth on valine, suggests the existence of a separate, specific transport mechanism for these ketoacids.

Mathieson & Catcheside (135) observed that arginine and lysine inhibited the growth of a histidineless mutant of *N. crassa*, and prevented the uptake and storage of exogenous histidine by the wild strain mycelium without interfering with its growth; in this case it is quite evident that the inhibitory action of the basic amino acids must be ascribed to their interference with the intracellular concentration of the exogenous histidine. An additional example of growth inhibition by interference with the transport of a required nutrilite across the cell membrane has been given by Mandelstam (136, 137) who showed that cadaverine inhibits the decarboxylation of lysine by intact cells, but not by acetone dried cells of *Bacterium cadaveris*, and interferes with the growth of a lysineless mutant of *E. coli*, but not with the growth of the wild strain.

It is apparent, in the light of all these observations, that many of the growth-promoting or growth-inhibitory effects of components of complex media may be due to specific interactions at the cell membrane; this possibility must be borne in mind whenever results obtained by the method of inhibition analysis are considered. A permeability barrier for L-glutamate in *L. arabinosus* is indicated by the report of Sondheimer & Wilson (138) that this organism can initiate growth on L-glutamic acid only when the pH of the medium is low, but can utilize glutamine over a wide range of pH. Acidic peptides, such as glutamyl-alanine behaved like glutamic acid and neutral peptides such as glutamyl-alanine, behaved like glutamine. Camien & Dunn (139) report L-aspartic acid to inhibit the growth of *L. arabinosus* on L- or D-glutamic acid, but not on glutamine; this observation may be explained by assuming that aspartic acid prevents the penetration of the cell by L- and D-glutamic acid.

The inhibitory effect of certain structural analogues of amino acids, for example, β-2-thienylalanine (an analogue of phenylalanine) and azatryptophan (an analogue of tryptophan), on the growth of *E. coli* appears to be a result of their incorporation into bacterial protein in place of the corresponding amino acids [Munier & Cohen (140); Pardee *et al.* (141)]. Dunn *et al.* (142) have shown that the inhibitory action of β-2-thienylalanine on the growth of *E. coli* is more readily reversed by peptides of phenylalanine than by phenylalanine itself; conversely, the inhibitory action of peptides of thienylalanine is more readily reversed by phenylalanine than by peptides of phenylalanine. Phenylalanine and one of its peptides together antagonized thienylalanine more effectively than either compound alone. According to the authors these results suggest a special metabolic role of phenylalanine

peptides which does not involve phenyalanine per se; however, in the light of the studies on membrane transport the results may be explained by assuming that phenylalanine and thienylalanine compete at the cell surface for passage, and inside the cell for incorporation into protein; and that their peptides compete with one another, but not with the amino acids, for passage and are converted inside the cell by hydrolysis to free amino acids. The results of the investigation by Dunn *et al.* (143) of the toxicity of thienylpyruvic acid and thienylalanine for *E. coli* and its reversal by phenylpyruvic acid may be interpreted correspondingly. A similar mechanism may explain the results obtained by Eiduson & Dunn (144) in their investigation of the action of inhibitors on the growth of *L. casei* on phenylalanine and its metabolic precursors phenyllactate and phenylpyruvate. Mandelic acid inhibited the growth of the organism on phenyllactate, but did not affect its growth on phenylalanine; *p*-fluorophenylalanine, on the other hand, inhibited the growth of the organism on phenylalanine but did not affect its growth on phenyllactate. Neither inhibitor was effective when phenylpyruvate was used. Apparently mandelic acid and *p*-fluorophenylalanine interfere specifically with the passage into the cell of the structurally related compounds phenyllactate and phenylalanine, respectively.

Another instance in which an inhibitor seems to interfere with the passage of a required nutrilite into the cell, is the inhibition of the synthesis of the "malic enzyme" in *L. arabinosus*, by the aspartic acid analogues, cysteic acid and β-hydroxyaspartic acid; the inhibition is reversed competitively by aspartic acid, and noncompetitively by glycylasparagine or asparagine [Ifland & Shive (145)]. Still another example of antagonism between structurally related amino acids which may be due to competition for a specific transport site is the inhibition of the growth of serine requiring hiochi bacteria by threonine and its reversal by serine [Teramoto *et al.* (146)].

In the cases discussed so far the inhibitor appears to prevent the uptake of a required nutrilite by the cell; the inhibition may be overcome competitively by increasing the concentration of the nutrilite or noncompetitively by replacing it with a derivative whose passage through the cell membrane is not prevented by the inhibitor. However, it may also be possible to block the entry of a growth inhibitor into the cell by supplying in the medium a compound structurally related to the inhibitor but innocuous to the cell; the compound capable of restoring growth in the presence of the inhibitor need not then be a metabolite. An example is the demonstration by Davis (147) that a number of amino acids reverse the inhibitory effect of high concentrations of homoserine on the growth of the wild strain of *E. coli*, but depress the growth-stimulatory effect of low levels of homoserine on the growth of a homoserine-requiring mutant; these opposite effects are explained by assuming that the exogenous amino acid in both cases blocks the passage of homoserine into the cell. In a similar manner, many structurally related amino acids overcome the inhibition of *E. coli* by high concentrations of aminobutyric acids, as reported by Friedman (148). Cohen & Rickenberg

(130) suggest that isoleucine plays such a role when it antagonizes the inhibitory action of valine on the growth of the valine-sensitive K-12 strain of *E. coli.* However, in this case, Umbarger & Brown (133) have shown clearly that isoleucine releases valine inhibition in a noncompetitive fashion. Moreover, not only isoleucine, but also its metabolic precursors, α,β-dihydroxy-β-methylvalerate, and α-ketomethylvalerate, which presumably do not compete with valine for membrane transport, are capable of reversing the valine inhibition [Umbarger & Adelberg (149)]. Exogenous isoleucine therefore does not seem to act by preventing the passage of valine into the cell: rather it appears that isoleucine is required because its synthesis is inhibited by valine.

Inducible transport systems.—A concentration mechanism for β-galactosides analogous to the one responsible for the transport of amino acids across the cell membrane, but specifically inducible by β-galactosides, has been discovered by Rickenberg *et al.* (150). The formation of the galactoside-concentrating system in *E. coli* occurs only in the presence of a β-galactoside and involves protein synthesis, as shown by its sensitivity to chloramphenicol. In all other respects, the system resembles closely the amino acid concentrating system just described. Methyl-β-D-thiogalactoside is concentrated intracellularly to the remarkably high level of about 5 per cent of the dry weight of the cell. It seems impossible that the cell should possess a sufficient number of receptor sites to retain so large a quantity of the β-galactoside, and the authors postulate that a specific "β-galactoside-permease," presumably located in the cell membrane, catalyzes the passage of the β-galactoside into the cell. The "permease" is distinct from the inducible β-galactosidase of *E. coli* but essential for its formation and its function in the intact cell. This is shown most clearly by the existence of two classes of lactose-negative mutants, which lack the galactosidase and the "permease," respectively. The former concentrate methyl-β-D-thiogalactoside intracellularly, but fail to produce the galactosidase. The latter do not concentrate the galactoside, form galactosidase only when the inducer is supplied at one hundred times the level necessary to induce the wild strain, and after induction fail to metabolize β-galactosides at a normal rate unless treated with toluene. It is thus evident that the "permease" is essential for the utilization of β-galactosides by the intact cell. The "permease" resembles many other inducible enzymes in that its formation, but not its action, is inhibited by glucose; it would appear that the formation of the galactosidase is less sensitive to glucose, because cells which have been exposed to the inducer in the absence of glucose for a period sufficient for the formation of the "permease" are capable of forming the galactosidase in the presence of glucose and retain this ability for a number of generations [Cohn (151)].

An inducible "permease" responsible for the concentration of citrate by *A. aerogenes* was discovered by Green & Davis (147). The "permease" is required for the utilization of citrate as energy source, or as nutrilite in the case of a mutant lacking the citrate-condensing enzyme. The properties of the

"citrate permease" appear to be the same as those of the other "permeases"; its formation involves protein synthesis and is inhibited by glucose and a few related sugars. The demonstration of an inducible "citrate-permease" is in excellent agreement with earlier studies by Kogut & Podovski (152) and by Barrett *et al.* (153) on the adaptation of *Pseudomonas fluorescens* to members of the tricarboxylic acid cycle. The results obtained by these investigators, recently reviewed by Stanier (154), had shown that the exposure of the cell to these compounds did not induce the formation of the enzymes of the tricarboxylic acid cycle which were already present in non-induced cells, but seemed rather to induce a system required for the transport of the compounds into the cell.

It is reasonable to suppose that the transport of most organic constituents of the culture across the cell membrane is mediated by specific "permeases," some constitutive and others inducible. Thus, Monod *et al.* (155) report the existence of a specific "glucose-permease" in *E. coli* and its absence in the glucose-negative mutant discovered by Doudoroff *et al.* (156, 157).

NUTRITION AND REGULATION OF METABOLISM

The results of starvation.—The study of microbial cells deprived of essential nutrients have provided valuable evidence for the role played by the environment in the regulation of metabolism. The effects of thymine starvation have been studied by Cohen & Barner (158, 159). Originally these investigators used a thymineless mutant of *E. coli*, but more recently they have induced thymine deficiency in the wild strain of this organism by inhibiting thymine synthesis with sulfanilamide. The inhibitory effect of sulfanilamide on the growth of the organism can be overcome according to Rutten *et al.* (160) by supplying the metabolites whose synthesis requires coenzymes that contain folic acid namely, methionine, serine, xanthine, and thymine. Cohen & Barner (159) found that the incubation of the organism in a glucose-mineral salts medium containing sulfanilamide and a mixture of these metabolites without thymine results in the death of the cells, that is, in the loss of the ability to reproduce when thymine is added after a period of starvation corresponding to the time required for one cell division. The same results had been obtained earlier when the thymineless mutant was incubated in a medium containing glucose and ammonia, but no thymine.

The thymine-starved cells continue to synthesize protein and RNA in the absence of thymine and may be induced to form adaptive enzymes. It appears that this cytoplasmic growth in the absence of DNA synthesis is responsible for the death of the cells, since death can be prevented by withholding either methionine or a purine base from the sulfanilamide-inhibited, thymine-starved cells. It seems likely that the lethal lesion is due to damage to the bacterial DNA during unbalanced growth; thus Weinberg & Latham (161) and Coughlin & Adelberg (162) report that the frequency of mutations increases during thymine starvation.

An inhibition of DNA synthesis other than by thymine deprivation may also result in unbalanced growth and death of the cells. Barner & Cohen (163) exposed *E. coli* to low doses of ultraviolet irradiation sufficient to inhibit DNA synthesis and permitted the cells to recover in liquid media. In a glucose-mineral salts medium capable of supporting the growth of the cells, rapid restoration of viability (about 20 min.) was followed by a secondary death of the cell population during a period (40 min.) sufficient for about one cell division, irrespective of the presence or absence of thymine. In media not capable of supporting the growth of the cells, either because of the lack of a carbon source, or because of the presence of the amino acid antagonist, 5-methyltryptophan, the initial restoration of viability was not followed by this secondary death. On the other hand, Doudney (164) has reported that the recovery of DNA synthesis in *E. coli* from a block produced by irradiation with x-rays requires tyrosine or phenylalanine, tryptophan, glutamate or aspartate, serine or glycine, uracil, and guanine. The author interprets these results as indicating that protein and RNA synthesis are required for the resumption of the synthesis of DNA. In this case such synthesis apparently does not lead to the death of the cell by unbalanced growth.

The results of studies with amino acid, purine, or uracil requiring mutants of *E. coli* deprived of their essential nutrilite have demonstrated a close link between protein synthesis and RNA synthesis. In general, no net synthesis of either macromolecule can be demonstrated in the starved cells which, however, remain viable [Pardee & Prestidge (165)]. An exception is the methionineless mutant discovered by Borek *et al.* (166, 167). Methionine-starved cells of this mutant continue to synthesize RNA in the absence of any net synthesis of protein. They remain viable, but recover their ability for protein synthesis much less readily when supplied with methionine after starvation than methionine-starved cells of another mutant apparently blocked in the same biosynthetic reaction, which do not synthesize RNA during starvation. The link between the synthesis of RNA and that of protein is also reflected in the observation of Ben Ishai & Volcani (168) that, during thymine starvation, the ratio of protein and RNA synthesized remains constant under different conditions affecting the rate of their synthesis. It is possible to dissociate RNA synthesis from protein synthesis with chloramphenicol, which inhibits protein synthesis, but not RNA synthesis (169, 170). It has now been reported independently by Pardee & Prestidge (165) and by Gros & Gros (171) that in amino acid-less mutants of *E. coli* the synthesis of RNA without concomitant protein synthesis in the presence of chloramphenicol proceeds only when the essential amino acid is provided. These results seem to indicate that RNA synthesis demands a complete array of the amino acids required for protein synthesis.

Nitrogen-starved ("resting") cells have long been used in the study of the degradation of carbon compounds, and their ability to assimilate a portion of the proffered carbon compounds as polysaccharide has been established

by Clifton and his co-workers (172). Trevelyan & Harrison (173) report that the type of polysaccharide formed by yeast cells during the anaerobic fermentation of glucose is controlled by the presence of a nitrogen course in the growth medium: nitrogen-starved cells assimilate a portion of the glucose as trehalose, glycogen, mannan and glucan; addition of a source of nitrogen inhibits the formation of the energy reserve carbohydrates, trehalose and glycogen, but does not interfere with the synthesis of the structural carbohydrates, mannan and glucan.

The effect of exogenous metabolites on their endogenous synthesis.—In a recent study Warner (174) has compared the utilization of ammonia for the synthesis of amino acids by *E. coli* and by *Leuconostoc mesenteroides* each growing in a medium containing glucose, ammonia labelled with N^{15}, and a mixture of amino acids and peptides. Neither *E. coli,* which is nutritionally independent nor *L. mesenteroides* which requires 18 amino acids, utilized the available ammonia for protein synthesis except for incorporation into amides. It appears that when an exogenous supply of amino acids is available, *E. coli* does not exert its ability to synthesize these compounds *de novo* from the products of glucose degradation and ammonia, but utilizes the components of the medium to the same extent as does *L. mesenteroides.* These results confirm earlier observations that the addition of an amino acid, a purine, or a pyrimidine, to growth media of *E. coli* and related organisms, frequently results in the preferential incorporation of the exogenous metabolite into the protein and nucleic acid of the cell with the almost complete suppression of its biosynthesis *de novo.* This effect has been the basis of a fruitful method ("isotopic competition") for the study of the biosynthesis of amino acids, purines, and pyrimidines [Roberts *et al.* (175)].

The inhibitory effect of exogenous metabolites on their own endogenous biosynthesis is also evident in auxotrophic mutants which accumulate the substrate of the blocked reaction, or compounds derived from it, in the culture fluid. The excreted material is found, in an amount that generally far exceeds the amount of the nutrilite required for full growth, in cultures containing the nutrilite in an amount insufficient for more than about half-maximal growth. The excretion of the normal intermediates of pyrimidine biosynthesis, orotic acid, dihydroorotic acid, and ureidosuccinic acid, by pyrimidineless mutants of *A. aerogenes* and *E. coli,* occurs only after the added nutrilite has been exhausted from the medium by the growth of the organism [Yates & Pardee (81); Brooke *et al.* (176)]. Experiments with a mutant requiring uracil as well as histidine showed that the formation of orotic acid, which occurred in the absence of growth in a medium devoid of uracil and of histidine, was inhibited without restoration of growth, by the addition of uracil to the medium (176). Similar observations indicate corresponding effects of valine and of adenine or guanine on the excretion of a α-ketoisovaleric acid and of 4-amino-5-imidazole carboxamide in valine-isoleucine and purine requiring mutants, respectively [Adelberg & Umbarger (177); Gots (47, 178)]. Other examples include the inhibition of the excretion of 4-amino

imidazole-riboside in a purineless mutant of *E. coli* and biotin-requiring yeast by purines (48, 102) and the inhibition of the excretion of xanthosine in a guanineless mutant of *A. aerogenes* by guanine (52). A tryptophanless mutant of *E. coli* was found to excrete indole in a medium devoid of tryptophan [Gibson *et al.* (179)]. Novick & Szilard (180) used the chemostat to study the excretion of another indole derivative, presumably indole-3-glycerol (34), by a tryptophanless mutant of *E. coli.* They found that the organism could produce this compound about four times as fast as it would be required to supply the cell with the tryptophan necessary for growth at an optimal rate, and that its production was strongly inhibited by an exogenous tryptophan level above the low value of 1 μg./l.

These results indicate that microorganisms have the capacity to produce purine and pyrimidine nucleotides and amino acids considerably faster than is necessary for growth at an optimal rate, and that the level of these compounds in the cell controls the rate of their synthesis. The mechanism of this control by negative feedback has been elucidated in two instances. Umbarger (21) has shown the irreversible deamination of L-threonine to α-ketobutyric acid to be an essential step in the biosynthesis of L-isoleucine in *E. coli.* He has also been able to demonstrate that the L-threonine dehydrase found in cells grown in a glucose-mineral salts medium is inhibited by L-isoleucine; the inhibition is competitive and the enzyme possesses about one hundred times as much affinity for the inhibitor, isoleucine, as for the substrate, L-threonine. This inhibition of threonine deamination explains the sparing effect of isoleucine on threonine utilization in a threonineless mutant. Yates & Pardee (181) have demonstrated the inhibition of the formation of ureidosuccinic acid, an early precursor of pyrimidines, from aspartic acid and carbamylphosphate, by cytidine-5′-phosphate and by cytidine in extracts of *E. coli.* The inhibition by uracil and by cytosine of the excretion of pyrimidine precursors discussed earlier appears to result from their conversion to cytidine-5′-phosphate. The intracellular level of a protein- or polynucleotide-building block, such as isoleucine or cytidine-5′-phosphate, will depend on the rate of its synthesis *de novo,* on the rate of its uptake from an exogenous supply, and on the rate of its incorporation into the polymer. The results obtained by Umbarger and by Yates & Pardee indicate that the inhibitory effect of isoleucine or cytidine-5′-phosphate on an essential step in their biosynthesis will be released only when the intracellular pool of these metabolites has been depleted by incorporation into protein or nucleic acid and is not being replenished from an exogenous supply. The results discussed earlier which indicate that the biosynthesis of most amino acids, purines, and pyrimidines is inhibited by the addition of these compounds to the growth medium suggest that mechanisms similar to the ones discovered by Umbarger and by Yates & Pardee may, in general, regulate the synthesis of essential metabolites.

The metabolic regulation of enzyme synthesis.—The most striking example of a specific effect of a component of the growth medium on the enzymatic

composition of the cell is the induction of enzyme formation. The role of the inducer, generally the substrate of the enzyme or a compound structurally related to the substrate, has been discussed in several recent reviews [Pollock (182); Monod (183)]. The experiments of Monod and his co-workers on the induced biosynthesis of β-galactosidase in *E. coli*, on which many of the present concepts of inducible enzyme formation are based, were carried out under conditions of gratuity, that is, under conditions in which the organism derived no apparent advantage or disadvantage from the presence of the inducer in the medium and of the enzyme in the cell. This was made possible by the discovery that certain compounds, for example methyl-β-D-thiogalactoside, were excellent inducers of the enzyme, but did not interact with the enzyme as substrates or inhibitors and were not attacked by any other enzyme of the organism. It could be shown that the synthesis of β-galactosidase under gratuitous conditions had exactly the same nutritional requirements as the synthesis of the other proteins of the bacterial cell (a source of carbon and of energy, a source of nitrogen and, in mutants, the appropriate nutrilite), and that the rate of β-galactosidase formation was directly proportional to the rate of synthesis of cell protein. Moreover, it could be shown by the use of radioactive tracers that the formation of β-galactosidase involves the synthesis *de novo* of the enzyme protein from small precursors.

There are, however, cases in which the nutritional requirements for the formation of an inducible enzyme are more complex than the nutritional requirements for growth. Thus, Dagley (184) has reported that *E. coli* possesses citridesmolase activity after growth in a medium containing citrate, glucose, and peptone, but not when any one of these three components is omitted. However, cells grown in a glucose-citrate-mineral salts medium acquire the ability to dissimilate citrate upon addition of peptone much more rapidly than cells grown without citrate. Dagley & Sykes (185) have now observed that the enzyme activity is associated with a component of *E. coli* extracts which sediments in the ultracentrifuge at 12 to 16 S. Extracts of cells grown without citrate do not show this component upon ultracentrifugation, whereas extracts of cells grown in a citrate containing medium without peptone, or of cells grown in the citrate-glucose-peptone medium and subsequently incubated in a medium devoid of peptone, are enzymatically inactive, but do possess the component sedimenting at 12 to 16 S. The authors suggest that the formation of the bulk of the enzyme molecule occurs in the presence of citrate and does not require peptone, but for the activation of the enzyme molecule the presence of peptone is necessary. The requirement for amino acids in the induced formation of formic hydrogenlyase in *E. coli* may perhaps be explained in a similar manner (186, 187). Umbarger & Brown (188) report that *E. coli* contains two distinct L-threonine dehydrases which are also active against L-serine. One of them is formed when the organism is grown in a minimal medium containing glucose as the sole source of carbon and energy and is essential for the biosynthesis of L-isoleucine (see pages 224 and 240 of this review); the other, which appears to

be identical with the enzyme discovered by Wood & Gunsalus (189), is formed in large amount only when the organism is grown under anaerobic conditions in a rich medium containing tryptone and yeast extract, but no glucose.

In other instances, the production of constitutive enzymes in a medium adequate for growth may be specifically prevented by a component of the medium. Thus, Monod and his co-workers (190, 191) have shown that *E. coli* grown in the presence of methionine or tryptophan lacks enzymes responsible for the biosynthesis of the appropriate amino acid, and Adelberg & Umbarger (177) reported that the level of the alanine-α-keto-isovalerate transaminase of *E. coli* is depressed by growth in a medium which contains valine, the product of this enzyme. Another example of the inhibition of the formation of enzymes by products of their action may be found in the observation by Umbarger (192) that extracts of *E. coli* grown under anaerobic conditions were capable of catalyzing the production of citrate from acetylphosphate and oxalacetate when the growth medium contained glucose as the only source of carbon, but not when it contained casein hydrolyzate and yeast extract in addition to glucose. It would appear that the presence of compounds such as glutamate and aspartate in the rich medium prevented the synthesis of this enzyme system which, in the case of the cells growing on glucose alone, is essential for their biosynthesis. Vogel (193) has recently investigated a similar phenomenon in the biosynthesis of arginine by *E. coli:* the production of acetylornithase, which catalyzes a step essential for the biosynthesis of arginine, was found to be prevented by the presence of arginine in the growth medium, and this inhibitory effect of arginine could not be overcome by acetylornithine, the substrate of the enzyme. Maas & Gorini (194, 195) have found arginine to inhibit the formation of two additional enzymes, ornithine transcarbamylase and argininosuccinase, which are essential for its biosynthesis.

In an earlier section of this review one mechanism by which a metabolite can control the rate of its synthesis has been discussed: the inhibition of the *action* of an enzyme catalyzing an early irreversible step in its biosynthesis. The observations discussed in the preceding paragraph indicate the existence of a second control mechanism: the inhibition by the metabolite of the *formation* of enzymes catalyzing essential steps in its biosynthesis. According to this concept the level of biosynthetic enzymes in the cell will be controlled by the intracellular concentration of their final products. This is borne out by the observation of Gorini & Maas (195) that the arginine-requiring mutant of *E. coli* grown in the chemostat at an arginine level insufficient for an optimal rate of growth was twenty-five times as rich in ornithine transcarbamylase as the wild strain grown without arginine. The mutant was not able to produce the enzyme when growth was prevented by lack of arginine, presumably because this amino acid is absolutely required for protein synthesis.

Purines and pyrimidines do not appear to be quite as essential for protein

synthesis. It is therefore possible to demonstrate the preferential synthesis of certain enzymes, that is, their formation with relatively little concomitant synthesis of other cell protein, during purine or pyrimidine starvation. In this manner the inhibitory effect of guanine and of uracil on the formation of enzymes involved in their biosynthesis could be demonstrated. A guanine-requiring mutant of *A. aerogenes* which is genetically incapable of producing xanthosine-5′-phosphate aminase (57) produces very little inosine-5′-phosphate dehydrogenase, the enzyme catalyzing the preceding step in guanine synthesis, during growth in the guanine containing medium. The rapid formation of inosine-5′-phosphate dehydrogenase begins after the exhaustion of the guanine from the medium and proceeds without measurable net synthesis of protein until the specific activity of cell-free extracts is almost thirty times as high as that of extracts of the wild strain grown in a medium without guanine. The formation of the enzyme in the absence of growth appears to involve the synthesis of protein, as shown by its dependence on a source of carbon and of nitrogen, and its inhibition by chloramphenicol [Levin & Magasanik (196)]. Similar results were obtained by Pardee (197) who showed that during pryrimidine starvation, pyrimidineless mutants of *E. coli* produced some of the enzymes involved in pyrimidine biosynthesis (ureidosuccinate synthetase, dihydroorotase, and dihydroorotate dehydrogenase) with very little net synthesis of protein. The activity of these enzymes reached levels between ten and one hundred times as high as those found in the wild strain.

The preferential synthesis of inducible enzymes also may occur under conditions of starvation. In fact, most of the early work on the synthesis of inducible enzymes in yeast and bacteria was done with resting cells, that is, cells deprived of a source of nitrogen and of a source of energy other than the one that may become available through the action of the enzyme induced on the inducing substrate. Rickenberg & Lester (198) reported that β-galactosidase is formed without a measurable net increase in cell protein when *E. coli* is exposed to an inducer of the enzyme in a medium which does not contain another source of carbon and energy; similar results have been obtained by Løvtrup (199). It has been shown in several instances that, under these conditions of starvation, the synthesis of the inducible enzymes depended on the utilization of endogenous nitrogen and energy sources of the cell. Thus, inhibition of energy metabolism by 2,4-dinitrophenol in bacteria, and interference with the utilization of the amino acids of the intracellular pool in yeast, prevents the formation of inducible enzymes (129, 200). The synthesis of inositol dehydrogenase by resting cells of purine- or pyrimidineless mutants of *A. aerogenes* proceeds in the absence of the required nutrilites (201). Other cases, however, have been reported in which amino acid, purine, or pyrimidine starvation resulted in the complete loss of the ability of mutants of *E. coli* to produce inducible enzymes [Monod *et al.* (202); Wainwright & Nevill (203)]. An explanation for this apparent discrepancy was offered by Pardee (204), who showed that pyrimidine-starved mutants of

E. coli could form β-galactosidase in the absence of an exogenous energy source, but not when such an energy source as, for example, glycerol or glucose was provided; his assumption that the formation of the inducible enzyme involves the degradation and resynthesis of bacterial RNA is supported by his observation that incorporation of labelled inorganic phosphate into nucleic acids occurs during pyrimidine starvation. The seemingly paradoxical finding that addition of an exogenous source of carbon and energy prevents the synthesis of the inducible enzyme indicates, according to Pardee, that under these conditions the limited amount of pyrimidine available is used up in the synthesis of other cell protein. The syntheses of different inducible enzymes are not equally affected by nitrogen starvation. Wainwright & Nevill (205) report that nitrogen-starved cells of *E. coli* incubated with the appropriate inducing agent and with an energy source (maltose or glucose) cannot produce β-galactosidase, but readily produce tetrathionate reductase and nitrate reductase. Amino acid-starved mutants of *E. coli* failed to produce nitrate reductase, but a uracil-starved mutant of this organism could produce a limited amount of this enzyme (203).

The significance of the observations on the preferential synthesis of enzymes becomes clearer if one considers them in conjunction with studies on the inhibitory effect of glucose on the formation of many bacterial enzymes. In bacteria and yeast, inducible enzymes, whose production is suppressed by glucose include: amino acid deaminases [see review by Gale (206)]; tryptophanase (207); histidase (208); enzymes involved in the degradation of carbohydrates [see review by Monod (209)]; and the "permeases" responsible for the membrane transport of β-galactosides (150) and of citrate (147). Several compounds closely related to glucose, such as galactose, gluconic acid, mannitol, and fructose may exert an effect which is similar, but less marked and less general (207, 208). Glucose does not affect the formation of all inducible enzymes; for example, nitrate reductase (205), tetrathionate reductase (205), most amino acid decarboxylases (206), penicillinase (210), and formic hydrogenlyase (187) are generally produced in glucose-containing media. In a recent study Davies (211), by using the chemostat, has shown that glucose affects the production of the constitutive enzyme invertase in *Saccharomyces fragilis;* the highest enzyme levels were found in cells whose growth rate had been restricted by supplying glucose at a concentration lower than 0.001 per cent. Interestingly enough, the formation of the enzyme was also inhibited by its substrate, sucrose, except at low concentration. Presumably the hydrolysis of sucrose by the enzyme provides more glucose than is removed by the metabolism of the cells. A similar effect of glucose on the production of lactase in *S. fragilis* has also been observed by Davies (212). The production of an extracellular β-galactosidase by *Ophiostoma multiannulatum* is likewise inhibited even by low levels of glucose [Hofsten (213)].

The physiological significance of the inhibitory effect of glucose on the

formation of certain enzymes was demonstrated by the study of a histidineless mutant of *A. aerogenes* and of a tryptophanless mutant of *E. coli* (208). It was shown that the presence of glucose in the growth medium inhibits the formation of histidase and of tryptophanase, respectively, and thus prevents the destruction of the essential amino acid.

The mechanism by which glucose exerts its inhibitory effect on the biosynthesis of certain inducible enzymes by *A. aerogenes* was investigated by Neidhardt & Magasanik (214, 215). The enzymes studied were inositol dehydrogenase, glycerol dehydrogenase, and histidase. The latter is the first of a series of enzymes degrading histidine via urocanic acid to ammonia, formamide, and glutamic acid (216). Glucose apparently does not prevent the entry of the inducing agent into the cell, since histidine degradation continued without the formation of new enzyme in histidine-grown cultures transplanted into a medium containing glucose and histidine. Moreover, it was found that the gratuitous production of inositol-dehydrogenase in the absence of the inducer, *myo*-inositol, which occurred when inositol-grown cells were cultured in a medium containing histidine or glycerol as the source of carbon, was inhibited by glucose; upon exhaustion of the glucose from the medium, the gratuitous synthesis of the enzyme was resumed at its original rate. It would appear, therefore, that glucose interferes with the production of the enzyme rather than with the process of induction. The inhibitory action of glucose could not be overcome by enriching the medium with mixtures of amino acids, purines, pyrimidines, and vitamins. Interestingly enough, the inhibitory effect of glucose on the biosynthesis of histidase could be reversed when the production of this enzyme in the presence of glucose was made a prerequisite for growth. This was accomplished by substituting histidine for ammonium sulfate in the glucose-mineral salts growth medium of the wild strain or *A. aerogenes*, and also by substituting histidine for glutamate in the glucose-ammonium sulfate-mineral salts growth medium of the glutamic acid-requiring mutant of *A. aerogenes* described by Gilvarg & Davis (40). These observations demonstrate that glucose permits histidase biosynthesis when the action of this enzyme system furnishes a product (ammonia and glutamate, respectively) necessary for growth and not obtainable from the degradation of glucose. Conversely, glucose inhibits histidase biosynthesis when the products of the enzyme action merely augment the supply of metabolites resulting from glucose degradation. These results agree with an earlier observation by Dawes (217), who found that *E. coli* could grow in a medium containing serine as the only source of nitrogen, and glucose. The cells grown under these conditions, in contrast to those grown in complex glucose-containing media, were capable of deaminating serine. However, such a reversal of the inhibitory effect of glucose does not occur in every case where it is essential for growth. The glutamic acid-deficient mutant of *A. areogenes* can utilize citrate in place of glutamate when it possesses the transport system required for the uptake of exogenous

citrate, but glucose inhibits the synthesis of this transport system even in a medium containing citrate but no glutamate, and thus prevents the growth of the mutant [Green & Davis (147)].

The aerobic degradation of glucose by *A. aerogenes* differs from that of other substrates in one remarkable respect. It proceeds at a rate at least double that commensurate with the growth rate and the total crop of cells. The extremely large amount of glucose metabolized is reflected in the level of labile phosphate present in the cells which is considerably higher than that present in cells grown on other sources of carbon (215). Similarly, it appears that the metabolism of glucose in the presence of an ammonium salt results in a plentiful supply of nitrogenous organic compounds (218). It may therefore be postulated that the observed effect of glucose on enzyme synthesis is the reflection of a feed-back mechanism, analogous to the ones discussed earlier, by which the intermediary metabolites control their level in the cell. Glucose is so rapidly metabolized by an enzyme system always present in the cells that its intermediary products ("energy rich" and other metabolically active carbon and nitrogen compounds) are formed faster than they can be removed by anabolic reactions. This accumulation seems to prevent the synthesis of those enzymes whose products would only augment the already large metabolic pools (215). This hypothesis is supported by an examination of the enzymes whose synthesis is prevented by glucose; as a rule, their physiological function is to supply energy by the degradation of carbohydrates and of amino acids, or nitrogen by the deamination of amino acids. In contrast, the enzymes whose formation is insensitive to glucose do not seem to be active in energy metabolism (see page 244 of this review). Furthermore, the formation of β-galactosidase is inhibited by an exogenous source of energy, such as glycerol, in uracil-starved cells of *E. coli* but not in growing cells (see page 243 of this review). It is evident that when protein formation is severely restricted by the lack of uracil the metabolites formed by the degradation of the carbon source are not rapidly removed by anabolic reactions, and many consequently prevent the formation of "glucose-sensitive" enzymes. On the other hand, formation of nitrate reductase and tetrathionate reductase, both "glucose-insensitive" enzymes, proceeds readily in nitrogen-starved cells in the presence of an exogenous energy source.

At present it is hard to visualize how the end-products of anabolic and catabolic reactions can control the synthesis of the enzymes responsible for their formation. It is evident that a highly integrated process such as the production of microbial protoplasm requires accurate and efficient control mechanisms. Microbiologists have become aware of the importance of these problems and have found in the study of bacterial nutrition an important approach to their solution.

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METABOLISM OF CARBOHYDRATES AND RELATED COMPOUNDS[1,2]

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Continued inquiry into the pathways and enzymes of carbohydrate metabolism in microorganisms again has enriched our knowledge and further shaped our concepts of this basic function of cells. As in the past the diversity of fermentative and oxidative types as well as the unique properties of the microbial cell have made the microorganisms a fruitful source of new phenomena concerning carbohydrate metabolism. Much of the 1956 literature represents extensions and detailed investigation of areas whose parameters were reported in past years. In a few cases the key experiments. a culmination of a long series, were reported. New trends have appeared, however. In the past, this inquiry has in one manner or another sought: (*a*) to elucidate the means by which the various pathways and reactions serve the energy demands of cells; (*b*) to show how these routes furnish required intermediates for biosynthesis; and (*c*) to understand the biochemical tests widely used in the classification of microorganisms. Now another major motivation is evident, namely the desire to relate the vast amount of knowledge concerning carbohydrate metabolism to larger questions of cellular physiology, in particular to enzyme induction, to structure and organization, and to transport activities. This trend with respect to metabolism in general is amply documented by the Henry Ford Hospital International Symposium entitled, *Enzymes: Units of Biological Structure and Function* (1) which is available in book form.

Reviews of carbohydrate metabolism by Korkes (2) and by Dickens (3) were published this year. These give a more general treatment in that the material is not restricted to microorganisms. Alexander's (4) review on the *Localization of Enzymes in the Microbial Cell* contains much information as to localization of enzymes of carbohydrate metabolism. For convenience this review is organized around the framework of the major pathways, with the reactions converting other carbohydrates and related compounds to intermediates common to a major system, the pathways of electrons, and the metabolism of organic acids being considered as branches of the main routes.

[1] The survey of the literature pertaining to this review was concluded in December, 1956.

[2] The following abbreviations will be used: ADP (adenosine diphosphate); AMP (adenosine monophosphate); ATP (adenosine triphosphate); CoA (coenzyme A); DPN (oxidized diphosphopyridine nucleotide); DPHN (diphosphopyridine nucleotide, reduced); TPN (oxidized triphosphopyridine nucleotide); and TPNH (triphosphopyridine nucleotide, reduced).

Pathways of Glucose Metabolism

Embden-Meyerhof glycolytic system.—The organism which possesses only the anaerobic glycolytic mechanism for carbohydrate degradation is proving to be exceptional. The majority of organisms, even fermentative types, are endowed with more complicated machinery. For instance, the fermentation of glucose, labeled in carbon atoms 1, 2, or 3 and 4, by *Clostridium perfringens* [Paege, Gibbs & Bard (5)] has confirmed the enzymatic data that the Embden-Meyerhof pathway functions in this *Clostridium*. However, the fermentation of glucose-1-C^{14} and glucose-6-C^{14} yielded the unexpected result that the specific activity of the methyl group of ethanol was diluted in excess of the expected 50 per cent whereas the activity of the acetate methyl group was near the expected value. Similar data were obtained with respect to the carbinol group of ethanol when glucose-2-C^{14} was fermented. If acetate and ethanol arose from a common precursor, the labeling should have been the same in each of these products. Similarly, Wood, Stjernholm & Leaver (6) found that glucose fermentation by propionic acid bacteria is a complex process. A major part of the glucose was metabolized via the glycolytic route. Yet, the high labeling of the CO_2 from glucose-1-C^{14}, the conversion of carbons 3 and 4 to acetate, and carbons 2 and 3 to propionate and succinate indicated that another route was involved. These studies have been verified in part by the finding [Wood, Kulka & Edson (7)] that extracts of *Propionibacterium shermanii* and *P. arabinosum* randomize the isotope from glucose-1-C^{14} into all positions of the products and that CO_2 is produced from C-1 of glucose. Reazin & Gibbs (8) reported that *Ochromonas malhamensis* ferments 1 mole of glucose to 1.6 moles of CO_2, 1.75 moles of ethanol and 0.18 mole of lactate. Glucose-1-C^{14} and glucose-6-C^{14} yielded methyl-labeled ethanol. In the presence of arsenite the pyruvate which accumulated from glucose-2-C^{14} was labeled exclusively in the α carbon whereas that from glucose-1-C^{14} was methyl-labeled. CO_2 arising from glucose oxidation was unlabeled. Hence it was concluded that the Embden-Meyerhof pathway is the main pathway of glucose dissimilation in this protozoan.

Of the glycolytic enzymes, the hexokinase of *Clostridium oedematiens* [Shemanova & Bogoveshchenskii (9)] and an inducible glucokinase [Sato, Takemori & Ebata (10)] of a bacillus have been purified and studied as to activator and substrate specificity. A Sauternes yeast which selectively ferments fructose was shown by Sols (11) to contain a hexokinase which phosphorylates both glucose and fructose. The results are interpreted as indicating that a transferring agent common to glucose and fructose functions prior to phosphorylation, and that this agent has a greater affinity for fructose. A specific fructokinase has been found in a fructose-adapted mutant of *Pseudomonas saccharophila* by Palleroni, Contopoulou & Doudoroff (12); however, fructose-6-phosphate rather than fructose-1-phosphate was formed. In addition, a mannose isomerase was present. In contrast, the wild type grown on maltose contained neither mannose isomerase nor fructokinase, but phosphorylated mannose to mannose-6-phosphate. Fructose-1,6-diphosphate aldolase has been purified from *Aspergillus niger* until electrophoretically

homogeneous by Jagannathan, Singh & Damodaran (13). It resembles yeast aldolase rather than muscle aldolase in that metal ions are required for activity. Inhibitions by metal-binding agents were reversed by zinc, manganese, iron, and cobalt ions. A crystalline phosphoglyceric acid mutase has been prepared by Rodwell, Towne & Grisolia (14) from bakers' yeast. Hexokinase and triose phosphate dehydrogenase also have been detected in *Endomyces vernalis* [Bruchmann (15)].

Hexose monophosphate pathways.—Kitos, King & Cheldelin (16) have compared the rate of $C^{14}O_2$ production from uniformly and specifically labeled glucoses by *Acetobacter suboxydans* and found a rapid and successive elimination of carbon atoms 1 and 2 and a slow release of carbon 6. This was interpreted as resulting from a cyclic oxidation of glucose-6-phosphate, as earlier postulated by Horecker (17) and by Racker (18).[3] There was also a significant release of carbon atoms 3 and 4 as CO_2. This was presumed to result from another route of carbohydrate utilization. Since β-labeled pyruvate did not yield $C^{14}O_2$ or labeled cells, it was concluded that the tricarboxylic acid cycle did not function in glucose oxidation by this organism. In a different type of experiment Bokman *et al.* (19) showed that pentose was formed when *Malleomyces pseudomallei* was incubated with glucose or gluconic acid. In addition, labeled glucose monophosphate, and phosphogluconate were formed from labeled glucose whereas labeled ribulose and ribose gave rise to $C^{14}O_2$. Anaerobic respiration or growth was not demonstrated. It was therefore concluded that a hexose monophosphate pathway operates in this organism.

Damodaran, Jagannathan & Singh (20) have followed the content of hexokinase, phosphohexose isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglyceromutase, enolase, phosphopyruvate kinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in *A. niger* with respect to culture age during the citric acid fermentation. Maximum activity occurred between three and five days. Since these enzymes are not unique to the glycolytic system, but are also part of the hexose monophosphate pathway it is a matter of conjecture as to the pathway to which the level of these enzymes should be related. A similar array of enzymes was demonstrated in extracts of *Myocobacterium smegmatis* by Volk & Myrvik (21). Phosphofructokinase was not found, however. Therefore, it was concluded that the Embden-Meyerhof pathway was not operating and that glucose oxidation proceeded via the hexose monophosphate route. Doudoroff *et al.* (22) presented evidence that mutants of *P. saccharophila*, which grow on glucose or fructose or both as the source of carbon, phosphorylate fructose. Further, fructose utilization involves glucose-6-phosphate, 6-phosphogluconate, and 2-keto-3-deoxy-6-phosphogluconate as intermediates.

[3] This process involves the oxidation of glucose-6-phosphate to 6-phosphogluconate and pentose phosphate and the resynthesis of hexose monophosphate by the action of transketolase and transaldolase. During a single turn of the cycle one carbon dioxide is released and two oxidations occur. By further cycling, carbon atoms 2 and 3 yield CO_2 successively.

Glucose-6-phosphate dehydrogenase from *A.niger* has been purified sixtyfold by Jagannathan, Rangachari & Damodaran (23). The preparation was free of phosphoglucoisomerase, phosphoglucomutase, hexokinase, and 6-phosphogluconate dehydrogenase. McNair Scott (24) has investigated the levels of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in *Escherichia coli* as a function of growth conditions. Cells grown aerobically or anaerobically on glucose or gluconate, or aerobically on arabinose or lactate contained twice as much glucose-6-phosphate dehydrogenase as 6-phosphogluconate dehydrogenase. Infection with certain bacteriophages did not alter the amount of extractable activity. When *E. coli* was grown in the presence of dinitrophenol there was a two to sixfold increase in glucose-6-phosphate dehydrogenase activity but no change in 6-phosphogluconate dehydrogenase (25).

Multiple pathways.—Many experiments with labeled glucose were designed to elucidate the pathways of glucose utilization, and in some cases to assess the degree to which one pathway of the several present functions in the intact organism. Thus Wang *et al.* (26), by comparing the specific activities of the CO_2 produced from specifically labeled glucoses by bakers' yeast, have estimated that 87 per cent of the glucose was metabolized via the Embden-Meyerhof pathway and tricarboxylic acid cycle, whereas 13 per cent was consumed by the hexose monophosphate route. Similar studies made with *Streptomyces griseus* [Wang, Bialy & Gilmour (27)] led to the conclusion that the glycolytic system and the tricarboxylic acid cycle are the predominant routes. The labeling of the products was such, however, that a condensation of CO_2 with a 3-carbon unit appeared to be of importance. Cochrane & Hawley (28) reached a similar conclusion in an extension of their earlier studies with *Streptomyces coelicolor*. Ribose-5-phosphate yielded ribulose, sedoheptulose, triose, fructose, and glucose monophosphates. In addition, oxidation of ribose-5-phosphate was dependent upon TPN. Evidence also was presented that fructose-1,6-diphosphate can be dephosphorylated and oxidized via the hexose monophosphate route. Heath, Nasser & Koffler (29) found that under anaerobic conditions *Fusarium lini* converts carbon atoms 3 and 4 of glucose-3,4-C^{14} to CO_2 and carbon 1 of glucose-1-C^{14} to the methyl group of ethanol. Aerobically carbon atom 1 yielded CO_2 more rapidly than the others. Thus, the Embden-Meyerhof system operates anaerobically, and another route, presumably the hexose monophosphate pathway, functions aerobically.

In contrast, Heath & Koffler (30) reported that *Penicillium chrysogenum* utilizes the hexose monophosphate system to the extent of 61 per cent; 34 per cent of the glucose is utilized by the hexose diphosphate pathway. In partial confirmation of this fact, Sih & Knight (31) have found that *P. chrysogenum* contains the enzymes of the Embden-Meyerhof scheme with the exception of phosphohexokinase. In addition hexose, heptose, and pentose phosphates were isolated by ion exchange chromatography. The glyceraldehyde-3-phosphate dehydrogenase may require TPN rather than DPN as the hydrogen acceptor. Bernstein, Sweet & Foster (32) isolated

ribonucleic acid from *E. coli* grown on specifically-labeled glucose substrates. The similarity of the label in the ribose to that of the glucose used indicated that ribose was synthesized predominantly by a loss of C-1 from hexose.

The effect of bacteriophage infection upon the pathways employed by *E. coli*, studied with labeled glucoses, has been summarized by Cohen (33). Cells infected with a T-even bacteriophage, in addition to displaying fundamental differences in nucleic acid metabolism, metabolize less glucose via the hexose monophosphate route and more by the anaerobic glycolytic mechanism than do normal cells.

An unusual fermentation by an osmophylic yeast has been found by Spencer & Sallans (34) to convert as much as 60 per cent of the glucose to polyhydric alcohols including, glycerol, erythritol, D-arabitol, and mannitol. In further studies [Spencer *et al.* (35)] glucose-1-C^{14} yielded terminal-labeled glycerol, 1,5-labeled D-arabitol, methyl-labeled ethanol, and succinate with 30 per cent of the label in the carboxyl groups and 70 per cent in the methylene carbons. With glucose-2-C^{14}, the glycerol was labeled in carbon 2 whereas the arabitol was labeled in carbon atoms 1, 2, and 4. The succinate label was reversed with respect to that obtained in the glucose-1-C^{14} fermentation. The authors point out that this distribution of carbons can be explained by the combined operation of both Embden-Meyerhof and phosphogluconate pathways with transketolase playing an important part in D-arabitol formation.

Gluconic acid fermentation by *Streptococcus faecalis*, studied by Sokatch, Prieto & Gunsalus (36), yielded 0.5 mole of carbon dioxide and 1.5 moles of lactate per mole of gluconate fermented, with the remainder of the carbon being accounted for as formate, acetate, and ethanol. Gluconate-1-C^{14} yielded $C^{14}O_2$ and carboxyl-labeled lactate whereas gluconate-6-C^{14} yielded only methyl-labeled lactate. From gluconate-2-C^{14} the lactate was labeled in all positions with the carbinol carbon being labeled 2 to 3 times higher than the other two positions. It was postulated that gluconate is phosphorylated to 6-phosphogluconate. Half of the latter is then oxidized to pentose phosphate and CO_2 whereas the other half is converted to 2-keto-3-deoxy-6-phosphogluconate which is cleaved to pyruvate and glyceraldehyde-3-phosphate as in the pseudomonads. In support of this postulate, cell extracts were shown to contain gluconokinase, 6-phosphogluconate dehydrogenase, enzymes which form heptulose phosphate from pentose phosphate, and 2-keto-3-deoxy-6-phosphogluconate aldolase. 6-Phosphogluconate dehydrase and transaldolase were not detected, however. VanDemark & Wood (37) found that extracts of *Microbacterium lacticum* oxidized a wide variety of phosphate esters of the glycolytic and hexose monophosphate pathways. The fermentation of hexose and pentose phosphates was sensitive to iodoacetate. In addition, a large number of glycolytic enzymes as well as TPN-linked glucose-6-phosphate and 6-phosphogluconate dehydrogenases, transketolase and transaldolase were demonstrated. In a similar study, VanDemark & Fukui (38) demonstrated the ability of gluconate-grown *Propionibacterium pentosaceum* to oxidize certain phosphate esters of the glycolytic and hexose mono-

phosphate route. Gluconokinase, glycolytic enzymes, and TPN-linked glucose-6-phosphate and 6-phosphogluconate dehydrogenases were found. 6-Phosphogluconate and ribose-5-phosphate yielded sedoheptulose-7-phosphate, fructose-6-phosphate, and pyruvate. Zajic, DeLey & Starr (39) obtained evidence based upon enzyme content that *Corynebacterium insidiosum* contains a hexose monophosphate pathway and glycolytic system. It is not known, however, whether all of the glycolytic enzymes are present. Tricarboxylic acids were oxidized by whole cells and aconitase and isocitric dehydrogenase were demonstrated in extracts. An inventory of enzymes in *Trichophyton mentagrophytes* by Jensen, Altschuller & Bard (40) revealed the presence of enzymes associated with the hexose monophosphate and hexose diphosphate routes as well as with the tricarboxylic cycle. Additional information was given as to the coenzyme specificity of the dehydrogenases present in these pathways. A review of the pathways for phosphate ester oxidation in *Pseudomonas fluorescens* by Wood (41) states that this organism is lacking in hexokinase, phosphohexokinase, and contains only traces of aldolase thereby suggesting that the glycolytic pathway is not involved in glucose oxidation. Instead two other routes accomplish the same purpose by producing pyruvate from 2-ketogluconate via an unknown route and from gluconate via 6-phosphogluconate and 2-keto-3-deoxy-6-phosphogluconate. In addition the enzymes of the pentose phosphate cycle are present.

Pathways initiated by glucose oxidation.—The oxidation of glucose prior to phosphorylation to yield gluconate and ketogluconate, previously found in *Pseudomonas* and *Acetobacter*, now appears to be of wider occurrence. Dalby & Blackwood (42) have found enzymes in particles of *Aerobacter aerogenes* which oxidize glucose to gluconate and 2-ketogluconate. A divalent metal ion was required. A wide variety of sugars and derivatives were oxidized, whereas the common phosphate esters such as glucose-6-phosphate were not. The product of the latter oxidations was not identified. The oxidation of glucose, gluconate, and 2-ketogluconate by *Serratia marcescens* consumes 3.0, 2.5, and 2.0 μM of oxygen per μM of substrate, respectively, and produces 2 μM of CO_2 [Wasserman, Hopkins & Seibles (43)]. Katznelson (44) surveyed 19 species belonging to *Xanthomonas*, *Pseudomonas*, *Agrobacterium*, *Erwinia*, and *Corynebacterium* for their ability to oxidize glucose, gluconate, and 2-ketogluconate. All species oxidized glucose, all but *Xanthomonas* oxidized gluconate, but only *Agrobacterium* and one *Erwinia* oxidized 2-ketogluconate. An enzyme system from *M. pseudomallei* was reported by Dowling & Levine (45) to oxidize glucose to gluconic acid and galactose to galactonic acid. As has been the case with *Pseudomonas* species soluble hydrogen-transport carriers such as DPN and TPN appeared not to be involved in these oxidations.

Recently, a different system for glucose oxidation to 2-ketogluconate has been described. Glucose oxidation in *Bacillus subtilis* was reported by Kunita & Fukumaru (46) to require DPN and form hydrogen peroxide. In addition, the oxidation by spore extracts of *Bacillus cereus var. terminalis*, studied by Halvorson & Church (47), was catalyzed by a soluble DPN-

requiring system. Thus the glucose and gluconate dehydrogenases of Gram-positive organisms appear to differ as to electron transport mechanism from those found in Gram-negative organisms.

A gluconic dehydrogenase has been purified from *Pseudomonas aeruginosa* by Ramakrishnan & Campbell (48) and studied as to specificity for substrate and electron acceptor. Experiments designed to reveal the nature of bound prosthetic groups which function in electron transport were not reported, however. A dehydrogenase which oxidizes 2-ketogluconate to 2,5-diketogluconate has been solubilized and purified from *Acetobacter melanogenum* by Datta & Katznelson (49). It has been assumed that the oxidation of glucose and gluconate to 2-ketogluconate results in a substantial energy yield via oxidative phosphorylation. Campbell, *et al.* (50) have attempted to measure this energy yield indirectly by comparing the amount of growth obtained on equivalent amounts of glucose, gluconate, and 2-ketogluconate added as the sole carbon source. Since the growth was identical for each substrate and since extracts failed to reduce DPN or TPN, or to form ATP during glucose oxidation, it was concluded that biochemical energy is not generated during glucose oxidation to 2-ketogluconate. It is possible, however, that a factor other than energy limited growth on these substrates. Under cuch conditions differences in energy yield might not appear as different amounts of growth.

The further metabolism of gluconate and 2-ketogluconate is initiated by specific kinases for each substrate. These kinases previously reported in *Aerobacter cloacae* by DeLey (51) have been studied in *P. fluorescens* by Narrod & Wood (52). 2-Keto-6-phosphogluconate, the product of 2-ketogluconokinase was isolated and identified chemically, whereas 6-phosphogluconate, the product of gluconokinase action was identified enzymatically. Crude extracts degraded 6-phosphogluconate to pyruvate and glyceraldehyde-3-phosphate, and 2-keto-6-phosphogluconate to 2 moles of pyruvate. 2-Ketogluconate-1-C^{14} and 2-ketogluconate-6-C^{14} and ATP incubated with crude extracts yielded carboxyl- and methyl-labeled pyruvate, respectively. This distribution is similar to that obtained for glucose degradation via 6-phosphogluconate and 2-keto-3-deoxy-6-phosphogluconate to pyruvate and glyceraldehyde-3-phosphate as is known to occur in *Pesudomonas*. DeLey (51) has established the existence of a TPN-linked 2-keto-6-phosphogluconate reductase in extracts of *A. cloacae;* 6-phosphogluconate was formed. In *A. cloacae*, the further metabolism of 6-phosphogluconate involves its conversion to pentose phosphate (51). In *P. fluorescens*, a similar reduction of 2-keto-6-phosphogluconate and degradation of 6-phosphogluconate via the Entner-Doudoroff pathway to pyruvate and glyceraldehyde-3-phosphate would explain the stoichiometry and labeling patterns obtained by Wood, Narrod & Hertlein (53).

The pathway followed by glucose carbons in the synthesis of shikimic acid, an intermediate in aromatic ring formation, has been investigated with position-labeled glucose by Srinivasan *et al.* (54). The labeling of the shikimic acid isolated suggested that the carboxyl group and carbon atoms 1 and 2 were derived from a 3-carbon glycolytic intermediate; carbon atoms 3, 4, 5,

and 6 apparently arose from a tetrose phosphate generated via the pentose phosphate pathway. Kalan *et al.* (55) found that extracts of an *E. coli* mutant converted phosphorylated carbohydrates to 5-dehydroshikimic acid whereas the same investigators (56) found that the same extracts convert sedoheptulose-1,7-diphosphate almost quantitatively to shikimic acid. DPN was required for this process. With sedoheptulose-4,5,6,7-C^{14}-1,7-diphosphate the label appeared without dilution in carbons 3, 4, 5, and 6 of shikimic acid.

Routes to Known Pathways

Glucosamine.—A series of papers dealing with the catabolism of glucosamine and related compounds has appeared. Wolfe, Morita & Nakada (57) reported that phosphorylation is the initial reaction in the metabolism of glucosamine by resting and dried cell preparations of *E. coli*. Since glucose was inhibitory it appears that glucosamine phosphorylation was catalyzed by hexokinase. The further degradation of glucosamine-6-phosphate by a ninefold purified enzyme containing phosphoglucomutase was reported by Wolfe & Nakada (58, 59); fructose-6-phosphate which accumulated was thought to be the deamination product. Roseman (60) and Comb & Roseman (61) have synthesized glucosamine-6-phosphate chemically and enzymatically (hexokinase) and isolated the crystalline barium salt. Incubation of this compound and other hexose phosphates with extracts of *E. coli* caused TPN reduction. Evidence was presented that glucosamine-6-phosphate oxidation occurs via fructose-6-phosphate and glucose-6-phosphate. A glucosamine-6-phosphate phosphatase has been purified seventy-fivefold from *Neurospora crassa* extracts by Blumenthal, Hemerline & Roseman (62). Its substrate specificity, lack of metal requirement, and pH optimum appear to differentiate this enzyme from other classes of phosphatases. The acetylation of glucosamine-6-phosphate by acetyl-CoA to form N-acetyl-glucosamine-6-phosphate is catalyzed by an enzyme from *N. crassa* which has been purified sixtyfold by Davidson, Blumenthal & Roseman (63). The enzyme was found in other molds, mammalian tissues, and in a Group A, hyaluronic acid-producing streptococcus. A phosphoacetylglucosamine mutase has been purified from *Neurospora* by Reissig (64). Purified fractions were obtained with ratios of phosphoglucomutase to phosphoacetylglucosamine mutase content varying from 400:1 to 0.2 to 1. At equilibrium, 14 per cent of N-acetylglucosamine-1-phosphate and 86 per cent of N-acetylglucosamine-6-phosphate were present. Either glucose-1,6-diphosphate or N-acetylglucosamine-1,6-diphosphate was required as a co-factor. In addition the following reaction was studied and N-acetylglucosamine-1,6-diphosphate was isolated:

$$\begin{array}{c} \text{Glucose-1,6-diphosphate} \\ + \\ \text{N-acetylglucosamine phosphate} \end{array} \leftrightarrows \begin{array}{c} \text{Glucose-1-phosphate} \\ + \\ \text{N-acetylglucosamine-1,6-diphosphate} \end{array}$$

The pathway of hyaluronate hexosamine synthesis in a Group A *Streptococcus* was studied by Lowther & Rogers (65). Cell-free extracts synthesized

glucosamine from glucose and glutamine in the presence of Mg^{++}, cysteine, and ATP. Wheat (66) reported that uridine diphosphoacetylglucosamine is metabolized by a Vi antigen-producing strain of *E. coli* to produce: (*a*) an increase in ultraviolet absorption with a maximum at 350 mμ; (*b*) a disappearance of the N-acetyl-glucosamine moiety of uridine diphosphoacetylglucosamine; (*c*) uridine diphosphate; and (*d*) a fluorescent substance containing the radioactivity from acetyl-labeled uridine diphosphoacetylglucosamine.

Polyols.—A series of polyol dehydrogenases of differing specificity and mechanism of action has been described. Arcus & Edson (67) studied a dehydrogenase from *A. suboxydans* which oxidized D-mannitol maximally at pH 5. The oxidation of certain other polyols indicated that the specificity followed the Bertrand-Hudson rule. The enzyme, termed "cytochrome-linked" D-mannitol dehydrogenase, was associated with a cytochrome-containing particle and did not require DPN for activity. A second, soluble dehydrogenase, named DPN-linked D-mannitol dehydrogenase possessed a different specificity pattern and an optimum activity at pH 8. A similar enzyme was also found in *Candida utilis*. Shaw (68) reported that DPN-linked polyol dehydrogenases can be induced in a *Pseudomonas* species. Growth on sorbitol and dulcitol induced a galactitol dehydrogenase which oxidized polyols of the L-threo configuration adjacent to the primary alcohol group. Thus, dulcitol was oxidized to D-tagatose. Dulcitol also induced the formation of a DPN-linked D-iditol dehydrogenase which oxidized polyols of the D-threo configuration adjacent to the primary alcohol group. Wolff & Kaplan (69) have recorded the ability of *E. coli* to grow on glucose, galactose, mannose, fructose, mannitol, sorbitol, D-ribose, and dulcitol. No growth was observed on L-sorbose, adonitol, L-arabitol, or erythritol. A D-mannitol-1-phosphate dehydrogenase was induced by a number of substrates, but was fourfold higher in cells grown on sorbitol and ten to fifteenfold higher in cells grown on mannitol. Extracts of cells grown on sorbitol, but not on mannitol or dulcitol reduced DPN with sorbitol-6-phosphate as the substrate. Extracts of dulcitol-grown cells reduced tagatose-6-phosphate with DPNH as the hydrogen donor to form, presumably, dulcitol-1-phosphate. D-Mannitol-1-phosphate dehydrogenase was purified thirtyfold and found to be specific for fructore-6-phosphate and DPNH [Wolff & Kaplan (70)]. Since these cells oxidized mannitol, a kinase for its phosphorylation to mannitol-1-phosphate was postulated. The phosphorylation of D-erythritol to D-erythritol-4-phosphate by extracts of *P. pentosaceum* has been reported by Shetter (71).

Pentoses.—Recent studies of pentose metabolism have centered around xylulose-5-phosphate which was shown to be formed from ribulose-5-phosphate [Dickens & Williamson (72)] and to be the substrate of yeast transketolase [Srere *et al.* (73)]. Stumpf & Horecker (74) prepared a specific xylulokinase from *Lactobacillus pentosus*, and isolated and characterized the phosphorylation product as xylulose-5-phosphate. It acted as a substrate for transketolase and was the substrate for a new enzyme, phosphoketopento-epimerase, which forms ribulose-5-phosphate by epimerization of the hy-

droxyl group on carbon 3. Phosphoketopentoepimerase was purified seven-hundredfold by Hurwitz & Horecker (75, 76) and the equilibrium established at 60 per cent xylulose-5-phosphate–40 per cent ribulose-5-phosphate. A method for the preparation of xylulose-5-phosphate from ribose-5-phosphate was given. With the aid of purified epimerase xylulose-5-phosphate was shown to be the obligatory donor for the liver and spinach transketolase reaction (Horecker, Smyrniotis & Hurwitz (77, 78, 79)]. With fructose-6-phosphate and triose phosphate as substrates, xylulose phosphate rather than ribulose phosphate was formed. Crystalline fructose-1,6-diphosphate aldolase was shown in earlier studies to be required for the transketolase reaction. This requirement was due to the need for epimerase which was present as a contaminant.

Trudinger (80) has observed the incorporation of $C^{14}O_2$ into the carboxyl group of 3-phosphoglycerate by extracts of *Thiobacillus denitrificans;* either ribose-5-phosphate and ATP or ribulose-1,5-diphosphate served as substrates. Extracts contained phosphoglycerokinase, triosephosphate dehydrogenase, aldolase and hexose diphosphate phosphatase. In the presence of phosphoglycerate, ATP and DPNH, hexose phosphates were synthesized. In addition enzymes of the pentose phosphate cycle were present. It was thus concluded that *T. denitrificans* synthesizes hexose phosphates from CO_2 by a cycle similar to that found in green plants. The carboxylation of ribulose diphosphate also has been shown in *E. coli* by Fuller (81). The greatest activity was obtained with cells grown on pentose in the presence of CO_2, thereby suggesting that ribulose diphosphate carboxylase synthesis can be induced.

The steps in D-xylose utilization by *Pseudomonas hydrophila* were studied by Hochster & Stone (82). Evidence was obtained for the synthesis of triose phosphate, fructose-6-phosphate and glucose-6-phosphate from xylose and ATP. Xylose was considered to be utilized via the transaldolase-transketolase pathway to the hexose phosphates, as suggested by earlier isotope data (83). In further studies [Stone & Hochster (84)] it was found that D-xylose and ribose-5-phosphate were oxidized by identical terminal pathways. With each substrate, the rate of utilization was more rapid with added DPN than with TPN. The overall oxidation was shown to proceed via glyceraldehyde-3-phosphate in the presence of DPN and probably via a hexose monophosphate cycle with TPN present. In this and other pentose-degrading systems an aldose-ketose interconversion is the first step. This generality now can be extended to the methyl pentoses. The interconversion of L-rhamnose and L-rhamnulose by an adaptive enzyme from *E. coli* was reported by Tecce, Di Girolamo & Lazzari (85) and by Wilson & Ajl (86). In the latter case the ability to utilize fucose appeared as a result of adaptation to rhamnose. In the presence of ATP, rhamnose evidently was phosphorylated. Similarly the adaptation to rhamnose utilization by *A. aerogenes,* studied by Garner & Fink (87), resulted in the acquisition of an ability to oxidize fucose. Green & Cohen (88) report that *E. coli* forms an adaptive isomerase for the L-fucose–L-fuculose interconversion. The same enzyme also catalyzed the interconversion of D-arabinose and D-ribulose. In view of

the above reports that rhamnose-adapted cells are adapted to fucose isomerization whereas fucose-adapted cells interconvert another pentose, it appears that a systematic study of the parallel rise in isomerase activity for various pentose-pentulose pairs and the specificity of the enzymes (purified) so induced would be fruitful. An unusual sugar containing D-rhamnulose, glucosyl rhamnuloside, has been prepared by Palleroni & Doudoroff (89) by the action of mannose isomerase and sucrose phosphorylase on sucrose and D-rhamnose. D-Rhamnulose, formed by mannose isomerase, acts as a glucosyl acceptor (like fructose) in the sucrose phosphorylase reaction. Palleroni & Doudoroff (90) report the presence of a mannose isomerase in fructose-utilizing mutants of *P. saccharophila*. In addition to the mannose-fructose interconversion, D-lyxose–D-xylulose, D-rhamnose–D-rhamnulose, and aldose (presumably D-glycero-D-manno-heptose)-sedoheptulose isomerizations also were observed. Baron (91) has found that transduction of xylose-negative *E. coli* to a xylose-positive strain involves the transfer of a gene controlling the formation of D-xylose isomerase.

It has long been known from fermentation studies with labeled pentoses that two distinct fermentative types exist. One is best explained by a cleavage between carbon atoms 2 and 3 to yield a 2-carbon unit (acetate) and a 3-carbon unit (lactate) more or less directly; the second type involves a shuffling of carbon atoms best explained by a conversion of pentose phosphate to hexose phosphate by transaldolase and transketolase and fermentation of the hexose monophosphate via the Embden-Meyerhof scheme. The existence of both routes has been further established by enzymatic studies. Heath, Hurwitz & Horecker (92) have demonstrated the presence of a ketolase in *L. pentosus*, which requires inorganic phosphate for the cleavage of xylulose-5-phosphate; glyceraldehyde-3-phosphate and acetyl phosphate are formed. Cocarboxylase and Mg ions are required and transketolase is not present in the extracts.

Wood (41) reported that extracts of *M. lacticum* contain both systems of pentose degradation. Anaerobically, ribose-5-phosphate yielded sedoheptulose-7-phosphate and hexose monophosphate indicating that transaldolase and transketolase were present. This sequence also was duplicated with charcoal treated preparations (DPN- and TPN-deficient) under aerobic conditions. With DPN added or with untreated extracts, however, ribose-5-phosphate was oxidized and pyruvate rather than sedoheptulose-7-phosphate or hexose monophosphate accumulated. Ribose-5-phosphate-1-C^{14} gave rise to unlabeled pyruvate, whereas ribose-5-phosphate-2,3-C^{14} yielded pyruvate labeled predominantly in the carboxyl group. Therefore it was postulated that pentose phosphate also can be cleaved to give free 2- and 3-carbon units. Acetate, glycolaldehyde, and erythrose or erythrulose were not produced, however.

One of three known routes of L-arabinose metabolism utilizes the second fermentation type involving the synthesis of hexose monophosphate by the transaldolase-transketolase route. Simpson, Kitch & Wood (93) found that extracts of *A. aerogenes* convert L-arabinose or L-ribulose plus ATP to ribu-

lose, ribose, heptulose, fructose, and triose phosphates. Either L-arabinose, L-ribulose plus ATP, or D-glyceraldehyde-3-phosphate caused DPNH formation whereas L-glyceraldehyde-3-phosphate did not. L-Ribulokinase was purified and ribulose-5-phosphate isolated, identified and shown to cause DPN reduction [Simpson & Wood (94)]. Therefore the conversion of L-ribulose-5-phosphate to a D-pentose phosphate was considered likely. Volk (95) reported that extracts of *P. pentosaceum* metabolized L-arabinose in a similar manner. Two different routes of L-arabinose metabolism also are currently under investigation. In one a mixture of uridine diphosphate xylose and uridine diphosphate arabinose (presumably L-arabinose) is formed by an enzyme preparation from mung beans from uridine triphosphate and α-xylose-1-phosphate [Ginsburg *et al.* (96, 97, 98)]. This suggests that the D-L conversion at carbon 4 is similar to the uridine diphosphoglucose–uridine diphosphogalactose reaction. In another system for L-arabinose metabolism, arsenite-poisoned *P. saccharophila* [Weimberg & Doudoroff (99)] or extracts therefrom, oxidize L-arabinose via L-arabono-γ-lactone to α-ketoglutarate. A DPN-linked dehydrogenase for L-arabinose oxidation, and a delactonizing enzyme have been demonstrated. The steps in the conversion of L-arabonic acid to ketoglutarate are undefined; however, either DPN or TPN serve as electron acceptors and the C_1 of arabonic acid becomes the α-carboxyl group of ketoglutarate. There was no evidence for phosphorylated intermediates or for the participation of well-known metabolites. Another pathway of L- and D-pentose interconversion has been found in guinea pig liver mitochondria by Hollman & Touster (100). L-Xylulose is reduced to xylitol by a TPN-specific dehydrogenase and xylitol is then oxidized at carbon 4 bv DPN-specific dehydrogenase to D-xylulose.

D-Arabinose and D-galactose oxidation by *P. saccharophila* has been observed by Doudoroff *et al.* (101) to follow the unique pattern typical of this organism. D-Arabinose, like L-arabinose, is oxidized by a DPN-specific dehydrogenase to D-arabono-λ-lactone which is then enzymatically hydrolyzed to arabonic acid. D-Arabonic acid is then dehydrated to form 2-keto-3-deoxy-D-arabonic acid [Palleroni & Doudoroff (102)] which is oxidized in the presence of DPN to pyruvate and glycolate. Similarly, galactose is converted to 2-keto-3-deoxy-D-galactonic acid which is then phosphorylated to 2-keto-3-deoxy-6-phosphogalactonate and cleaved to pyruvate and glyceraldehyde-3-phosphate. In conformity with the analogous mechanism for 6-phosphogluconate utilization the pyruvate carboxyl group arises from carbon 1 of the pentonic or hexonic acid.

Arnstein & Bentley (103) tested the ability of the eight pentoses to serve as substrates for kojic acid formation by *Aspergillus flavus* and *Aspergillus oryzae*. D-Ribose and D-xylose gave the best yields. L-Arabinose was less effective and the others gave little or no kojic acid. L-Rhamnose was a fairly good source for kojic acid whereas L-fucose was not. With D-ribose-1-C^{14} the radioactivity of carbon dioxide was low initially but increased to a maximum in three days. 80 per cent of the radioactivity of the isolated kojic acid was

in carbon atoms 1, 2 and 3. This fact was interpreted to indicate that a C_6 precursor was formed by transketolase and transaldolase which was further oxidized.

Miscellaneous carbohydrates.—Ebata, Sato & Bak (104) have found a highly specific kinase for sedoheptulose in extracts of a bacillus adapted to sedoheptulose. Cultures of *Alcaligenes faecalis* and *Aerobacter aerogenes* have been obtained by Hiatt & Horecker (105) which utilize D-erythrose. Extracts of *A. faecalis* required ATP for this process and catalyzed an erythrose-dependent exchange of P^{32} from ATP into sedoheptulose diphosphate. Cort *et al.* (106) have presented evidence for the formation and utilization of lactobionic acid by *P. chrysogenum*. Dickens & Williamson (107) have obtained a protein fraction from bakers' yeast which, in the presence of methylene blue, oxidizes L-glycerate at twice the rate of lactate oxidation. In addition, the pH optimum and Michaelis constants for glycerate and lactate oxidation are different. Further experiments are in progress to determine whether lactic dehydrogenase or another enzyme is involved. Payne (108) observed that glucose-grown *S. marcescens* oxidized pentoses rapidly but not glucuronic or galacturonic acids. Prolonged culturing on either uronic acid yielded cells which oxidized glucuronic acid rapidly and galacturonic acid slowly. Adapted dried cells converted glucuronate into a compound thought to be a 1,6 "ester"-linked dihexuronic acid. This compound also was utilized by dried cells.

Di- and polysaccharides.—A series of papers has appeared concerning the mode of action and specificity of levan sucrase. Hestrin, Feingold & Avigad (109) have found that all of the compounds with a terminal β-fructofuranosidic group (fr) linked to the anomeric carbon of an aldose-formed levan whereas those compounds with the same group linked to a carbinol carbon did not. Thus it was postulated that the former group has a bond with higher free energy (designated $fr\sim R$) than the carbinol linkages ($fr<R$). For the type $fr\sim R$, glucose, galactose, xylose, melibiose, and others serve as R. The same investigators (110, 111) report that levan sucrase of *Aerobacter levanicum* forms fructo-oligosaccharides from sucrose which are hydrolyzable by invertase but not by levan sucrase. The major product was 1^F-β-fructosylsucrose. Further transfructosylation forms the mono-, di-, and trifructosides of this acceptor. When fructose was the acceptor, inulobiose and levanbiose were formed. Transfructosylation to glucose yielded 2-, 3-, and 6-β-fructosyl glucoses. Hestrin *et al.* (111) also report that the action of levan sucrase upon raffinose forms levan, fructose, and melibiose. When glucose was present, however, sucrose rather than levan was formed. Dextran was formed in a mixture of dextran sucrase, levan sucrase, raffinose and glucose. The formation of various aldosyl-β-D-fructofuranosides with D-xylose, L-arabinose, D-glucose, D-galactose and melibiose was reported. Avigad *et al.* (112) have studied in detail the formation of α-D-xylopyranosyl-β-D-fructofuranoside. These and other findings have led to the suggestion (109) that the transformations catalyzed by levan sucrase involve a readily reversible reaction fol-

lowed by an essentially irreversible one as follows:

$$fr \sim R + \text{enzyme} \leftrightarrows fr \sim \text{enz} + R$$
$$fr \sim \text{enz} + \text{acceptor} \rightarrow fr < \text{acceptor} + \text{enzyme}$$

Water or a carbinol group is the acceptor for the second reaction. Peaud-Lenoel (113) reported that levan is fixed reversibly to specific areas of levan sucrase which are distinct from those where the donor of fructosyl is fixed. This conclusion is based upon the ability of levan to act as a noncompetitive inhibitor of sucrose hydrolysis and triose (trisaccharide) formation and as an activator for levan synthesis. Henis (114) has found a levan sucrase in *Corynebacterium* which is different than those hitherto described. Its formation was induced by growth on sucrose and organic nitrogen. Nitrate-reducing *P. fluorescens* strains were isolated by enrichment on levan by Fuchs (115). These strains formed a constitutive endocellular levan sucrase.

The synthesis of an α,3′-linked glucosyl disaccharide (3-α-D-glucopyranosyl-D-glucose) by the transferring enzyme from *A. oryzae* has been reported by Pazur, Budovich & Tipton (116). This compound was formed during the conversion of maltose into a series of α-1,6′-linked oligosaccharides. It was suggested that this type of action may account for the irregularities observed in starch, dextran, and pentosan. The effect of streptomycin upon *Acetobacter capsulatin* and its dextran-dextrinase system has been studied by Arnold & Hall (117). Sery & Hehre (118) have found in intestinal bacteria (bacteroides) a soluble enzyme system containing two dextranases which hydrolyze both 1,4- and 1,6-linkages of oligosaccharides and dextran.

The action of cellulase from *Myrothecium verrucaria* upon cellulose has been under investigation in a number of laboratories. Aitken *et al.* (119) reported that cell-free culture filtrates of this organism may contain β-1,4-glucosidases such as cellulase, cellobiase, a cellobiase transglucosidase activity, and-β-1,3-glucosidases which hydrolyze laminarin and laminaribose. The ability of both of these enzymes to hydrolyze the β-glucosans of barley was studied. The mechanism of *Myrothecium* cellulase action upon cellodextrin and cellotriose has been studied by Whitaker (120) and by Whitaker & Merler (121), respectively. By incubating a highly purified cellulase in the presence of cellobiose-C^{14}, the cellotriose and cellotetrose formed at various stages of the hydrolysis had less than 20 per cent of the cellobiose specific activity isolated at the same time. These and other data led to the conclusion that cellodextrin hydrolysis does not involve cleavage of cellobiose units from the ends of the chains, but rather a random cleavage. Using cellotriose labeled in the anhydroglucose unit at the reducing end, it was found that the glucosidic linkage at the nonreducing end was hydrolyzed 5 times faster than at the reducing end. The synthesis and secretion of invertase has been studied in relation to growth of *Myrothecium* by Mandels (122). Buston & Khan (123) report that extracts of *Chaetomium globosum* grown on cellobiose synthesize cellotriose and form a variety of β-glucose-linked saccharides from cellobiose. The phosphorolytic degradation of cellobiose by *Clostridium thermocellum* has been reported by Sih & McBee (124).

The factors affecting the production of pentosanases by *Bacillus pumilus* and *B. subtilis* have been studied by Simpson (125). The yields of extracellular pentosanase as a function of industrial sources of carbohydrate and nitrogen were reported. Inaoka & Soda (126) have prepared a crystalline xylanase from a bacillus. Calcium or magnesium is required for activity. Hydrolysis occurred in two phases, the first of which was accompanied by a rapid decrease in viscosity and hydrolysis of 9 per cent of the xylosidic linkages. Xylose di-, tri-, and oligosaccharides were produced but free xylose was not. Sorensen (127) was able to grow *Sporocytophaga myxococcoides* on a mixture of cotton wool and xylan but not on either substrate alone. Xylose, arabinose, and xylobiose accumulated. Cell material grown on cellulose or glucose and precipitated with acetone cleaved xylan. It was therefore concluded that the cellulase of this organism can cleave xylan. α-Amylase from *P. saccharophila* has been crystallized by Markovitz, Klein & Fischer (128) and some of its properties determined. Further studies by Sowden & Frankel (129) of mannan synthesis by *Torula utilis* grown on glucose-1-C^{14} have revealed that in addition to nonradioactive mannan, a second radioactive mannan could be isolated. Essentially all of the radioactivity occurs in C-1 of the D-mannose component. The radioactivity was identical to that of glucose C-1 in the glycogen fraction. From this and other data it was suggested that D-mannose arises by isomerization of D-glucose.

β-Galactosidase.—Due to the fact that β-galactosidase has become the model for studies of adaptive enzyme formation, its formation and behavior have been studied from a number of viewpoints. Landman & Speigelman (130) have defined the conditions for protoplast formation in *Bacillus megaterium* and investigated the induction of β-galactosidase by protoplasts. When 50 per cent of the ribonucleic acid was removed from noninduced protoplasts, enzyme synthesizing ability was lost. Cohn (131) has shown that the induction of galactosidase by methyl-β-D-thiogalactoside in *E. coli* was completely inhibited by glucose. Other data indicate that glucose blocks the synthesis of the "y" (inducer concentrating) system, not its function. Rickenberg & Lester (132) have demonstrated β-galactosidase formation without a corresponding increase in cell protein and termed this a process "preferential" synthesis. Lovtrup (133) has found that β-galactosidase synthesis in the presence of lactose occurs in the absence of endogenous nitrogen. This synthesis was decreased by starvation, extraction with water, or pretreatment with glucose. Synthetic capacity was recovered partially by adding alanine or ATP. Polglase, Peretz & Root (134) observed that the formation of β-galactosidase in dihydrostreptomycin-sensitive *E. coli* is inhibited by dihydrostreptomycin. An *E. coli* strain which requires the antibiotic for growth also requires it for β-galactosidase synthesis [Polglase (135)]. Aladjem *et al.* (136) separated the β-galactosidase from *E. coli* into three components which could be distinguished by electrophoresis on starch. These components from cells grown in lactose hydrolyzed both lactose and *o*-nitrophenylgalactoside. Two of the three components of cells unadapted to lactose, or adapted to lactose by a short incubation period with lactose, hydrolyzed

both substrates. The third component hydrolyzed lactose but not *o*-nitrophenylgalactoside. Neidhardt & Magasanik (137) also have observed the inhibitory effect of glucose upon the induction of myoinositol dehydrogenase, glycerol dehydrogenase, and histidase in *A. aerogenes*. Glucose did not prevent the entry of L-histidine (histidase inducer) into the cell. The control of enzyme synthesis by a feed-back mechanism sensitive to the level of metabolites was discussed as a possible cause of this inhibition [Neidhardt & Magasanik (138)].

Organic Acids

Pyruvate and lactate.—Mortlock & Wolfe (139) have fractionated the pyruvate-$C^{14}O_2$ exchange system present in *Clostridium butyricum* with protamine and ammonium sulfate and recovered 85 per cent of the activity. In the absence of phosphate, $C^{14}O_2$ exchange was dependent upon added CoA. With stoichiometric amounts, acetyl CoA, CO_2 and H_2 accumulated. C^{14}-acetyl-CoA exchanged with pyruvate at a much slower rate than did $C^{14}O_2$, whereas acetyl-C^{14}-adenylic acid and formate-C^{14} did not exchange with pyruvate. Lactate and pyruvate fermentation by dried preparations of a rumen microorganism was found by Peel (140) to form H_2, CO_2, and acetyl phosphate. Hydrogen formation was relatively insensitive to arsenite but was sensitive to dilution and inhibited by carbon monoxide. Riboflavin and 2,6-dichlorophenolindophenol acted as acceptors for a carbon monoxide insensitive oxidation of pyruvate which required heat stable cofactors for activity. The extracts contained phosphotransacetylase; thus acetyl CoA was considered to be the primary product, which in the intact organism is used in fatty acid synthesis. Dawes & Foster (141) have presented evidence that the route from pyruvate to ethanol in *E. coli* involves acetyl CoA and acetaldehyde as intermediates.

The oxidation of ethanol and acetaldehyde by *Acetobacter peroxydans* has been reported by Tanenbaum (142) to involve two TPN-specific dehydrogenases. The conversion of acetaldehyde to acetate was not CoA dependent. Extracts also contained TPNH oxidase, TPNH-cytochrome-*c* reductase, diaphorase, peroxidase, and enzymes for a hydrogen peroxide-consuming peroxidation of TPNH or reduced cytochrome-*c*. Acetate oxidation was thought to occur by a dicarboxylic rather than a tricarboxylic acid cycle. Atkinson (143) also studying *A. peroxydans* found that ethanol-grown cells oxidized ethanol, pyruvate, succinate, malate, fumarate, and oxalacetate, but not acetate, to CO_2 and water. Cell-free extracts oxidized ethanol rapidly to acetate, but the tricarboxylic acid cycle intermediates were oxidized only slowly. In contrast to the findings of Tanenbaum, the first step in ethanol oxidation was DPN-linked. DPN reduction was cyanide insensitive whereas DPNH oxidation was cyanide sensitive.

A comparison of pyruvate oxidation by *S. faecalis* and *Streptococcus mitis* has been made by Clapper & Meade (144). $MgSO_4$, thiamin, riboflavin, adenosine, and glutamate stimulated respiration of both organisms. Lipoic acid further stimulated *S. faecalis* respiration but not that of *S. mitis*. Lipoic

acid was not detected in extracts of *S. mitis*. Seventy-four strains of lactate fermenting spore-forming anaerobes, isolated from silages, have been characterized by Bryant & Burkey (145) as *Clostridium tyrobutyricum*.

Sutton (146) reported that the lactic oxidative decarboxylase of *Mycobacterium phlei*, which forms acetate and CO_2, is a multifunctional enzyme. Although flavin mononucleotide was the only prosthetic group found, H_2O_2 did not accumulate. This fact and the inability of carbonyl fixatives to inhibit CO_2 production led to the suggestion that the product of lactate oxidation is peroxidized on the enzyme surface to form acetate and CO_2. In similar studies Cousins (147) concluded that a purified lactic oxidase from *M. smegmatis* does not utilize flavinadenine dinucleotide as a prosthetic group. During the oxidation of 30 μM of lactate 1.2 μM of H_2O_2 and 0.9 μM of pyruvate accumulated. Possible mechanisms for the oxidation were discussed.

Juni & Heym (148) have found that under anaerobic conditions pyruvate oxidases from animal tissues and from *E. coli* produce *dl*-α-acetolactic acid and acetoin from pyruvate. Evidence was presented that the acetoin arose from a condensation of free acetaldehyde with "activated" acetaldehyde. Diacetylmethylcarbinol and acetoin were formed when diacetyl was the substrate. Dickens & Williamson (149) have found that erythrulose was produced by carboxylase action on hydroxypyruvate presumably by a similar mechanism. Slodki & Kalnitsky (150) reported that extracts of *Proteus morgani* formed *d*-α-acetolactate from pyruvate. Diphosphothiamin and CoA were required cofactors. However, acetolactate was not decarboxylated to acetoin. Another acetoin-forming system which involved the condensation of pyruvate and acetaldehyde was present in purified preparations.

Juni & Heym (151) have discovered in unidentified organisms which grow on 2,3-butanediol (2,3-BD) or acetylmethylcarbinol (AMC), a unique cyclic mechanism for fermenting these substrates to acetate. In this process diacetyl, diacetylmethylacarbinol (DAMC) and acetylbutanediol (ABD) are intermediates. The scheme below is reprinted from their article.[4] The enzyme responsible for converting diacetyl irreversibly into diacetylmethylcarbinol and acetic acid has been purified and its stoichiometry determined. Diphosphothiamin (DPT) and a divalent cation are required for activity. The enzyme did not degrade pyruvate [Juni & Heym (152)].

The mechanism of hydrogen formation and utilization continues to receive much attention. Peck & Gest (153) have published a new hydrogenase assay which is based upon the manometric measurement of hydrogen evolved with reduced methyl biologen as the donor. A series of organisms was examined by the "evolution assay" and by the ability to oxidize hydrogen in the presence of methylene blue. The same authors (154) reported that a cell-free hydrogenase from *Clostridium butylicum* which does not reduce methylene blue, has been purified about sixtyfold with the aid of the "evolution

[4] Figures 1 and 2 were originally published in the *Journal of Bacteriology* as cited in the references. Permission of the copyright owner and the authors to reproduce these is gratefully acknowledged.

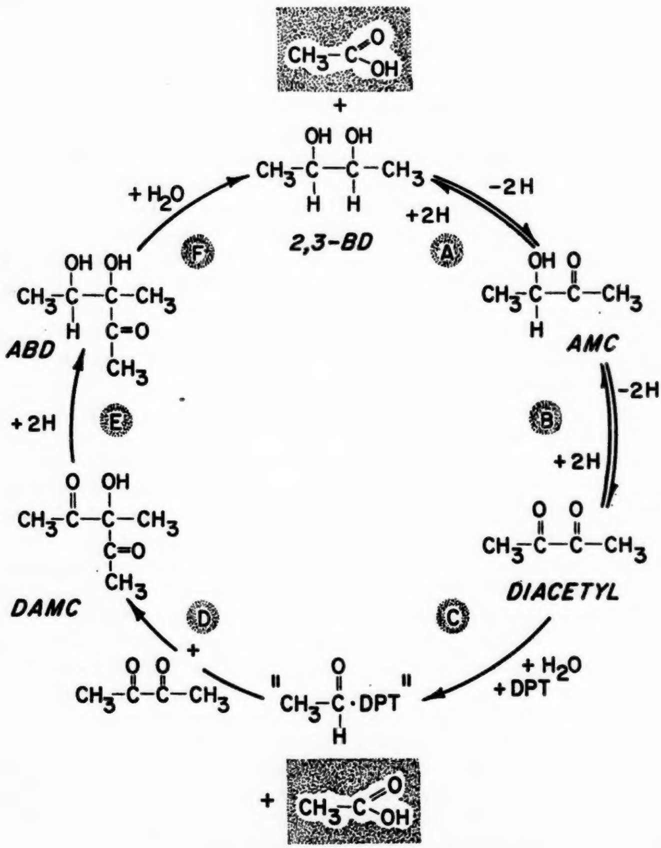

FIG. 1. Reactions of the 2,3-butanediol cycle.

assay." A marked stimulation of rate was obtained by adding Fe^{++}, MO_3^{-} and flavin to *o*-phenanthroline-treated and dialyzed preparations. Hydrogen evolution was completely inhibited by carbon monoxide. In line with an earlier suggestion [Peck, San Pietro & Gest (155)] it was postulated that ferrous ions act at the site of hydrogen activation, whereas molybdate and flavin participate in electron transfer to and from the primary site. The hydrogenase of *Desulfovibrio desulfuricans* has been purified fiftyfold by Sanada & Jagannathan (156). Methylene blue and benzyl viologen were reduced rapidly. Flavin mononucleotide and flavinadenine dinucleotide were reduced at 1 per cent of that rate whereas DPN, TPN, sulfate, and ferricyanide were not reduced. Fe^{++} or Fe^{+++} and cysteine or glutathione

acted as activators. Shug & Wilson (157) reported that extracts of *Clostridium pasteurianum* contain soluble enzymes which form acetyl phosphate, CO_2, and H_2 from pyruvate and contain a hydrogenase which reduces methyl or benzyl viologen, methylene blue and cytochrome-*c*. Fe^{++}, molybdate and methyl viologen revived the activity lost on dilution. Fe^{++} stimulated methyl viologen reduction whereas molybdate increased hydrogen production. Antimycin inhibited both processes but not pyruvate decarboxylation, the oxidation of H_2 by methyl viologen, or the evolution of hydrogen from reduced dye. Carbon monoxide completely inhibited viologen reduction by either pyruvate or hydrogen. The effects of nitric oxide, cyanide, heating and ammonium sulfate fractionation upon dye reduction and hydrogen evolution also were studied. Atkinson (158) reported that the hydrogenase of *A. peroxydans* utilized molecular hydrogen to reduce hydrogen peroxide, dyes, and oxygen but not to reduce viologens or methylene blue. Extracts reduced methylene blue and cytochrome-*c*, but not pyridine nucleotides. Although formate was oxidized and formic dehydrogenase was present, formic hydrogenlyase could not be demonstrated. In similar studies by Tanenbaum (159) it was found that deuterium exchange by cell suspensions did not occur at 30° C. and was slow at 37° C.; conversion of *para* to normal hydrogen did not occur. The kinetics of *para* hydrogen conversion and deuterium-water exchange catalyzed by suspensions and dried cells of *E. coli* and *Proteus vulgaris* have been studied by Couper, Eley & Hayward (160). The apparent energy of activation for the hydrogenase catalyzed exchange and the effect of water content of dried cells upon these processes was studied. Hydration of dried cells with D_2O gave lower activity and inactivated part of the hydrogenase by a different process than occurs with heat.

Wolin & Lichstein (161) have solubilized the particle-bound formic dehydrogenase of *E. coli* by treating the particles with sucrose, butanol, and lipase. Zn^{++}, Fe^{+++} or Fe^{++} stimulated the soluble enzyme whereas azide, cyanide, citrate, and versene were inhibitory. Of a large series of acceptors tested only methylene blue was reduced. The preparation did not contain hydrogenase. Stokes (162) reported that gas formation in *Salmonella* as in *E. coli* is due to the action of a hydrogenlyase which, although not present in anaerobically grown cells, is rapidly induced when glucose and amino acids are present. Anaerogenic strains cannot form hydrogenlyase and hence accumulate formate. Both types of *Salmonella* contain hydrogenase and formic dehydrogenase. Thus, it was concluded that other enzymes in addition to these function in the hydrogenlyase reaction. Orgel, Dewar & Koffler (163) have found that *Hydrogenomonas facilis* grown on $C^{14}O_2$ produces radioactive formate and doubly labeled acetate.

Tricarboxylic acid cycle.—By the use of mutants of *E. coli* and *A. aerogenes* which do not produce acetyl CoA-oxalacetate condensing enzyme, Gilvarg & Davis (164) found the tricarboxylic acid cycle to be the only significant route of acetate oxidation and of α-ketoglutarate synthesis. The "back to back" condensation of acetate to form succinate was excluded. These conclusions are based upon the fact that the mutants exhibit a nutritional re-

quirement for glutamate or α-ketoglutarate, oxidize succinate but not acetate, and oxidize glucose to a decreased extent. Goldschmidt, Yall & Koffler (165) also conclude from studies of radioactive acetate oxidation by *P. chrysogenum* that the tricarboxylic acid cycle is the main pathway.

The stimulation of oxygen uptake by whole cells and acetone-dried preparations of *Mycobacterium tuberculosis* by substrates of the tricarboxylic acid cycle and related compounds has been studied by Youmans, Millman & Youmans (166). After oxidation of radioactive acetate, tricarboxylic acid cycle intermediates as well as glutamate and aspartate were isolated by chromatography, thereby indicating that acetate oxidation occurs by way of the cycle. King, Kawasaki & Cheldelin (167) have presented evidence that *Acetobacter pasteurianum* contains an intact tricarboxylic acid cycle. The lack of biochemical similarity with *A. suboxydans* was pointed out. Crook & Lindstrom (168) found that *Rhodospirillum rubrum* and *Rhodospirillum palustris* contain enzymes of the tricarboxylic acid cycle whether grown under conditions of photo- or chemosynthesis. Campbell & Smith (169) have reexamined the tricarboxylic acid cycle in *P. aeruginosa* and found that pyruvate was oxidized without passage through oxalosuccinate and α-ketoglutarate as intermediates. This is due to the presence of isocitritase which cleaves D-isocitrate to succinate plus glyoxalate. Dagley & Walker (170) have demonstrated the accumulation of citrate and pyruvate during the oxidation in the presence of fluoroacetate of long chain fatty acids and aromatic compounds by a vibrio. It was concluded that widely divergent pathways of degradation converge upon the tricarboxylic acid cycle. Wiame, Rosenbloom & Bourgeois (171) have confirmed the theory that acidity inhibits bacterial growth by reducing the CO_2 content of the medium.This effect was overcome by adding tricarboxylic acid cycle intermediates and nucleic acid derivatives.

Smith, Stamer & Gunsalus (172) have extended their earlier studies of isocitritase and citritase to a comparison of the equilibria and energetics of these cleavage reactions. Both reactions are exergonic in the direction of synthesis, to the extent of 2000 and 200 cal., respectively. Since the reactions are second-order in the direction of synthesis and first-order in the direction of cleavage, a large concentration effect results in a considerable reaction in the direction of cleavage. Neilson (173) has found two *cis*-aconitate metabolizing enzymes in *A. niger*. One resembled aconitase in that citrate and isocitrate were formed from *cis*-aconitate whereas the second termed "aconitate hydrase" converted *cis*-aconitate only to citrate. Cells which accumulated citrate during growth on a defined medium did not contain either enzyme. As the manganese concentration was increased the dehydrase appeared, followed by aconitase at higher manganese concentrations. Wheat, Rust & Ajl (194) have determined the ammonium sulfate precipitation patterns of tricarboxylic acid cycle enzymes of *E. coli*. Purification of certain of these enzymes was reported. Goldman (175) has purified isocitric dehydrogenase from *M. tuberculosis* and demonstrated its similarity to that from

animal tissues with respect to metal ion activation, pH optimum, K_s values, and coenzyme specificity.

A soluble succinic dehydrogenase has been prepared from *Proteus vulgaris* by Kearney & Singer (176) by sonic oscillation. Cytochrome-*c* and conventional dyes did not serve as acceptors whereas phenazine methosulfate did. Other properties are described. A similar readily reversible dehydrogenase from bakers' yeast was solubilized and studied by Singer *et al.* (177). The enzyme has an iron:flavin ratio of 4. The soluble enzyme did not reduce methylene blue, 2,6-dichlorophenol indophenol, or cytochrome-*c*. Ferricyanide was reduced at 12 per cent the rate of phenazine methosulfate. The succinic dehydrogenase system of *Bacterium tularense* has been solubilized by Wadkins & Mills (178) and found to consist of several components. This conclusion was based upon finding fractions of differing heat and acid lability and cyanide sensitivity. In addition, Mg^{++} and Mn^{++} were required for indophenol reduction. Oxidase activity, which had been destroyed by dialysis, was restored by Mg^{++} or Mn^{++} and cysteine. Indophenol reduction and oxygen consumption were inhibited by atabrine, riboflavin, and flavin-adenine dinucleotide, thereby indicating that a flavin participates in these reactions. Reduction of phenazine methosulfate was not inhibited, however. Kusunose *et al.* (179) found that particulate succinic dehydrogenase of *Mycobacterium avium* required a soluble fraction or fumarase for a malonate insensitive reduction of 2,6-dichlorophenol indophenol or cytochrome-*c*. The aerobic oxidation of succinate was malonate-sensitive and required an oxalacetic decarboxylase (*Micrococcus lysodeikticus*) rather than fumarase for activity. The inactivating effect of hydrostatic pressure upon the succinic dehydrogenase of *E. coli* has been measured by Morita & Zobell (180).

Wood, Stjernholm & Leaver (181), and Leaver & Stjernholm (182) have found on incubating propionate-2,3-C^{14} and propionate-1,3-C^{14} with *P. arabinosum* that carbon atoms 2 and 3 of the reisolated propionate were randomized. A conversion of propionate to succinate or to a symmetrical C_3 compound was not involved in this process. Another experiment with propionate-1,3-C^{14} revealed that the labeling of the internal succinate, propionate, and malate was similar but different from the labeling in the external acids. It was postulated that two pools of succinate and propionate exist and that there is more than one pathway of propionate synthesis. The former authors reported that succinate was not cleaved to give two acetate molecules. Intercellular propionate was converted to intercellular succinate rapidly, a fact not observed by isotope carrier technique. In short-term experiments malate also acquired radioactivity at about the same rate as succinate. Elsden *et al.* (183) have isolated an organism from the rumen of sheep which is related to *Neisseria* or *Moraxella*. The fermentation of DL-lactate yielded hydrogen, carbon dioxide, acetate, propionate, *n*-butyrate, and *n*-valerate, whereas glucose or fructose yielded acetate, butyrate, *n*-valerate, and hexanoate. Growing cultures did not decarboxylate succinate. Delwiche *et al.* (184) have studied the formation of propionate and CO_2 by

P. pentosaceum and *Veillonella gazogenes*. The total reaction required CoA and ATP. Hydroxamate formation with succinate and fatty acids and ATP was demonstrated. Transuccinylase, acyl-CoA deaylase, succinyl-propionyl CoA transphorase, and acetyl-succinyl transphorase activities were demonstrated. In further studies, Phares *et al.* (185) found the following evidence that extracts of *P. pentosaceum* and *V. gazogenes* form a C_1 fragment from the γ-carboxyl of succinyl CoA which ultimately becomes CO_2: (*a*) nonstoichiometry between CO_2 and propionate production from succinate; (*b*) in the presence of malonate, the C_1 was found in the carboxyl of malate; (*c*) propionate exchanged with succinate by combination with C_1 arising from succinate decarboxylation; (*d*) with combinations of enzyme preparations

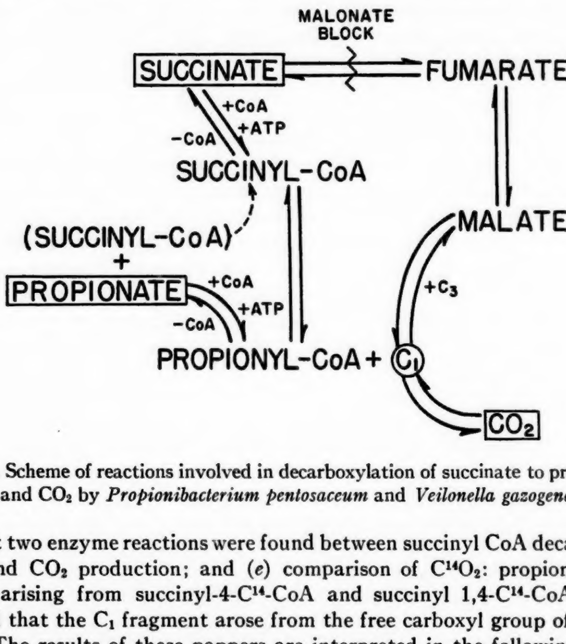

FIG. 2. Scheme of reactions involved in decarboxylation of succinate to propionate and CO_2 by *Propionibacterium pentosaceum* and *Veilonella gazogenes*.

at least two enzyme reactions were found between succinyl CoA decarboxylation and CO_2 production; and (*e*) comparison of $C^{14}O_2$: propionate-1-C^{14} ratios arising from succinyl-4-C^{14}-CoA and succinyl 1,4-C^{14}-CoA demonstrated that the C_1 fragment arose from the free carboxyl group of succinyl CoA. The results of these pappers are interpreted in the following scheme presented by Phares *et al.* (185).[4]

Stadtman (186) has found that the oxidation of propionate by extracts of *Clostridium propionicum* occurs with oxygen or dyes as electron acceptor as follows:

(*i*) Acetyl-P + propionate + CoA → propionyl-CoA + acetate + P_i
(*ii*) Propionyl-CoA ⇆ acrylyl-CoA + 2H
(*iii*) Acrylyl-CoA + NH_3 → β-alanyl-CoA

The acyl-CoA derivatives could be replaced by the corresponding acyl pantotheines. This formulation was supported by the following evidence: (*a*)

propionate oxidation required acetyl-P; (*b*) acetyl-P was not required for the oxidation of propionyl-CoA; (*c*) the reverse of a reaction similar to Equation ii occurred with reduced safranine and acrylyl pantotheine; and (*d*) with a partially purified enzyme and ammonium ion, acrylyl pantotheine disappeared; β-alanyl pantotheine was formed stoichiometrically and identified.

Wong & Ajl (187) reported the presence of a malate synthetase in *E. coli* which, in the presence of added transacetylase, converted acetyl phosphate and glyoxalate to malate; CoA was required. The counterpart of citritase or isocitritase, an enzyme which cleaves oxalacetate to oxalate and acetate has been found in *A. niger* by Hayaishi *et al.* (188). Manganese was the only required cofactor. Krakow & Barkulis (189) have found that *E. coli* extracts condense two molecules of glyoxylate to form hydroxypyruvate (or tartronic aldehyde) and CO_2. Manganese ions and thiamin pyrophosphate were stimulatory. Kimura, Katayama & Sasakawa (190) reported that the decarboxylation of oxalate by avian tuberculosis bacteria was accelerated by CoA and ATP. Hence the process was considered to involve oxalyl-CoA and formyl-CoA as intermediates. Active forms of acetate and formate were transferred to aromatic amines. Krampitz & Lynen (191) have isolated a bacillus which ferments *d*-tartrate. Evidence was presented that *d*-tartrate is converted to oxalacetate. La Rivière (192) also concluded that oxalacetate is formed anaerobically in tartrate decomposition by *Pseudomonas putida.* Induction experiments have revealed that *Pseudomonas putrefaciens* forms two distinct tartrate-attacking enzymes, one specific for the (+), the other specific for the (−) tartrate [La Rivière (193)].

Leaver (194) allowed *P. arabinosum* to ferment lactic acid-3-C^{14} in the presence of lactic aldehyde and found the reisolated lactic aldehyde to be labeled. In addition, lactic aldehyde was slowly converted to propionate. The acetoacetic decarboxylase in *Bacillus macerans* has been investigated by Barrett & Haynie (195) as to properties and factors influencing its formation. Pine & Barker (196) have further investigated the mechanism of methanogenesis by enrichment cultures of an acetate-fermenting, methane-producing organism using deuterium labeled acetate. It was found that the methyl group of acetate was converted to methane without loss of the hydrogen atoms.

Hydrogen Transport and Oxidative Phosphorylation

Rapid progress is being made towards characterization and reconstruction of electron transport systems. A number of cytochrome pigments have been prepared and their properties recorded. A cytochrome from *Chromatium* was purified by Newton & Kamen (197). This mesoporphyrin has an Fe:heme ratio of 2.0 to 2.7, and E'_0 of −0.04 volts and a molecular weight of 37,000. Two cytochromes, $-c_4$ and $-c_5$, were purified from *Azotobacter vinelandii* and their properties studied by Tissières & Burris (198) and Tissières (199). Pure $-c_4$ contains 0.46 per cent iron and has an E'_0 of +0.30 v. whereas the E'_0 for $-c_5$ is +0.32 v. Vernon (200) reported that *Micrococcus denitrificans* and *Pseudomonas denitrificans* contain a cytochrome-*c* which is

similar to its mammalian counterpart. Both organisms also contain cytochrome-*b* (b_1) with a protohematin prosthetic group. The cytochrome-b_1 was rapidly reduced by DPNH, and slowly by succinate. It reacts directly with oxygen and does not combine with cyanide. Similar studies have resulted in the preparation of three soluble cytochromes from an unidentified nitrate-reducing pseudomonad [Vernon (201)]; one is a cytochrome-*c*, which functions in cyanide-sensitive respiration between succinate or DPNH and oxygen. The second is a cytochrome-c_1 which functions between succinate and oxygen. The third component is similar to the cytochrome-b_1 above. Kamen & Takeda (202) have prepared cytochrome-*c* from *P. aeruginosa* with a purity of greater than 99 per cent. Its spectral behavior was similar to that of mammalian cytochrome-*c*. It was inactive in mammalian systems for DPNH or succinate oxidation but could serve as a substrate for reduction of oxides of nitrogen by bacterial extracts. This cytochrome-*c* also differs markedly in amino acid composition. A bifunctional hemato-hematin, cytochrome-c_3, has been isolated from *D. desulfuricans* and characterized by Postgate (203, 204). A cytochrome peroxidase has been purified from *P. fluorescens* by Lenhoff & Kaplan (205) which oxidizes pseudomonas cytochrome pigment and reduced 2,6-dichlorobenzenoneindo-3′-chlorophenol. Properties of a cytochrome-*c* also are described. Crystalline cytochrome-*c* has been prepared from yeast by Hagihara *et al.* (206) and its properties compared with cystalline cytochrome-*c* from beef muscle and heart (207).

The levels of cytochromes, cytochrome peroxidase, catalase, and "diaphorase," in *P. fluorescens* as a function of oxygen, iron, and molybdenum concentrations in the medium were studied by Lenhoff, Nicholas & Kaplan (208). More cytochrome pigments, cytochrome peroxidase, catalase, and less diaphorase were produced under low oxygen tension. The reverse was true with high oxygen tension. With iron deficiency, low oxygen tension decreased growth markedly, whereas high oxygen tension allowed normal growth. Low iron and low oxygen tension restricted the production of cytochromes and catalase whereas the nitrate reductase increased elevenfold. Diaphorase content also increased in iron deficiency. The same but greater effect was noted with molybdenum deficiency.

A cytochrome-b_2 has been isolated from bakers' yeast by Boeri & Tosi (209) which contains one flavin mononucleotide, one heme group, and eight iron atoms not bound to heme. In the presence of lactate and *o*-phenanthroline an active red compound is produced. The lactic dehydrogenase activity of this preparation is reversible with reduced flavin mononucleotide and pyruvate acting as substrates.

Dolin (210) has purified a DPNH-flavoprotein peroxidase, the first of its kind, from *S. faecalis*. It contains 0.66 per cent flavinadenine dinucleotide but no hemin compounds. DPNH combines with the peroxidase in such a way as to block the chemical reduction of the flavin. Other mechanism studies are reported.

Oxidative phosphorylation.—Rose & Ochoa (211) have observed a Mg^{++}-dependent phosphorylation of ADP coupled to the oxidation of several

substrates with oxygen by particles of *A. vinelandii*. Hydrogen, succinate, DPNH, and TPNH served as substrates. Owing to the lack of myokinase in the preparation it was shown that ADP but not AMP was the phosphate acceptor. Oxidative phosphorylation was observed in extracts of *M. phlei* and *Corynebacterium creatinovorans* by Brodie & Gray (212). P:O ratios greater than 1 were obtained with succinate, β-hydroxybutyrate, pyruvate, malate, fumarate, and α-ketoglutarate as substrates. ATP was formed with AMP as the acceptor. Methods of preparing the extracts were stressed and uncoupling agents were surveyed. Another paper by these authors (213) reported that both the soluble fraction and particles are required for the esterification of inorganic phosphate. Frenkel (214) has observed that partially purified preparations of *R. rubrum*, illuminated in the presence of inorganic phosphate, succinate or DPNH and magnesium, form ATP from ADP but not from AMP. Smith & Baltscheffsky (215) found that illumination of *R. rubrum* extracts or the addition of hydrosulfite, caused the appearance of an absorption peak at 434 mμ. The peak did not appear in the presence of ADP unless an inhibitor of phosphorylation was added. It was suggested that the system catalyzing photophosphorylation is different from the respiratory system. Photophosphorylation also has been observed in *Chlorobium* by Williams (216).

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NITROGEN METABOLISM[1,2]

With Special Reference to the Synthesis of Proteins and Nucleic Acids

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Papers on microbial metabolism appear regularly in some fifty scientific journals and the task of a reviewer today is no simple one. Many are capable of reading the newspaper while eating breakfast, but few have learned the art of reading scientific literature while working at the laboratory bench, and the time when a working scientist can produce a review accounted both critical and comprehensive is almost past. He is faced with the alternative of preparing an account of selected contributions in a restricted field or attempting a comprehensive catalogue of references. Although the former inevitably gives rise to criticism from those whose work is omitted, we have preferred to adopt this method rather than to attempt the latter which is so much more effectively done by an automatic punch card system (which we do not possess). In view of the fact that the last volume of this Annual Review contained an article on nitrogen metabolism in general, we have decided to confine our attention on this occasion to the later stages of protein and nucleic acid synthesis (the events taking place subsequent to the synthesis of amino acids and nucleotides) and to survey, in the main, contributions made to this field within the last two years.

Transport of Amino Acids

It has been established for some years that certain bacteria, particularly Gram-positive species, can accumulate high concentrations of amino acids in the free state (within the internal medium) and that this passage of amino acids into the cell requires an active transport mechanism (1). Some confusion was introduced by the demonstration by Britten (2) that glutamic acid diffuses freely into 35 to 40 per cent of the cell pellet when *Staphylococcus aureus* cells are suspended in a solution of the amino acid and then centrifuged

[1] The survey of the literature pertaining to this review was completed in December, 1956.

[2] The following abbreviations are used in this chapter: ADP (adenosine diphosphate); AMP (adenosine monophosphate); ATP (adenosine triphosphate); CDP (cytidine diphosphate); CMP (cytidine monophosphate); DNA (deoxyribonucleic acid); GDP (guanosine diphosphate); GMP (guanosine monophosphate); IDP (inosine diphosphate); IMP (inosine monophosphate); RNA (ribonucleic acid); TCA (trichloracetic acid); TP (thymidine 5′-phosphate); TTP (thymidine triphosphate); UDP (uridine diphosphate); UMP (uridine monophosphate).

[3] Our thanks are due to all the members of the Unit for Chemical Microbiology for their help in searching the literature and for the stimulation of their discussions.

down. It was not made clear at first that this permeable volume included the intercellular space, amounting to *ca.* 30 per cent of the cell pellet, and that the cells themselves were essentially impermeable to glutamic acid. Mitchell & Moyle (3) have now confirmed by direct measurement that the impermeable volume of the staphylococcus towards glutamic acid represents at least 90 per cent of the cell volume.

A further demonstration of the existence of a barrier to free penetration has followed from the studies of Rowlands and his colleagues (4) on the accumulation of free glutamic acid in *S. aureus* incubated with a variety of glutamic acid peptides. No peptide was found which would give rise to internal free glutamic acid in the absence of a source of energy. In the presence of glucose all α-glutamyl peptides or glutamic acid peptides tested gave rise to free glutamic acid within the cells, but at widely differing rates. There was no correlation between the rate of accumulation and the rate of hydrolysis of the peptide or its partition coefficient between *iso*-butanol and water, but there was a highly significant correlation between the rate of accumulation and the product (rate of hydrolysis × partition coefficient). The necessity for a partition coefficient factor indicates that penetration through a barrier is involved in the accumulation of free glutamic acid within the cells. Evidence that the barrier operates against glutamic acid itself is shown by the fact that the rate of internal accumulation is markedly greater when α-L-glutamyl-L-leucine or -L-valine is supplied as the external source than when glutamic acid itself is supplied. Diethyl glutamic ester was found to act as a source of internal free glutamic acid in the absence of a supply of energy and evidence was obtained that the ester penetrates into the cell more rapidly than it is hydrolysed within the cell, with the result that the ester accumulates within the cell. Kihara, McCullough & Snell (5, 6) have shown that the inhibitory action of a number of amino acid analogues is noncompetitively overcome by the presence of peptides containing the corresponding amino acids. Thus the growth of *Lactobacillus arabinosus* is inhibited by D-alanine which appears to prevent the assimilation of L-alanine; but D-alanine has no inhibitory action in the presence of L-alanyl peptides. The authors suggest that peptides pass across cell membranes and are converted into free amino acids within the cell; such a hypothesis is consistent with the results of Rowlands *et al.* (4).

An important contribution to our knowledge of the transport of amino acids in Gram-negative organisms has been made by Cohen & Rickenberg (7, 8) who have shown that *Escherichia coli* cells can effect a high concentration of amino acids across the surface membranes. In the case of valine a concentration gradient of 460 times across the cell surface has been measured. The process requires a source of energy, is inhibited by 2,4-dinitrophenol or sodium azide, and is very rapid, equilibrium across the surface being reached in approximately 1 min. at 37°C. The concentrating mechanism is independent of protein synthesis and is not affected by the presence of chloramphenicol or 5-methyltryptophan. The concentration process is competitive

in cases of amino acids of related structure; thus, competition occurs between valine, leucine, and isoleucine, but not between valine and phenylalanine or methionine or any valine-containing peptide. If valine is concentrated within cells which are then incubated further in the presence of isoleucine or leucine, the internal valine is partly displaced by the second amino acid. Phenylalanine is competitively displaced by *p*-fluorophenylalanine, but not by isoleucine, D-phenylalanine or L-phenylserine.

The authors suggest that their results are explained by the presence in the cell surface of concentrating mechanisms of limited specificity possessing properties similar to those of enzymes. They relate their findings to those (described below) of Monod and his colleagues on the presence of "permeases" involved in the specific penetration of galactosides and glucosides into microbial cells. The results with valine, leucine, and isoleucine explain a number of findings in bacterial nutrition. It has long been known that these three amino acids are mutually antagonistic (9 to 12); in particular, many strains of *E. coli* are inhibited by the presence of valine and the inhibition is released by the further addition of isoleucine. Cohen & Rickenberg point out that the addition of isoleucine will displace valine from within the cells and so decrease the toxic concentration. In the case of a valine-dependent strain they showed that displacement of the essential amino acid by isoleucine or leucine resulted in inhibition of growth; similarly, the displacement of isoleucine by valine inhibited the growth of an isoleucine-requiring strain. In the case of a strain (K12S) which was resistant to the presence of valine they showed that, although isoleucine displaced valine from the cell, growth was independent of the presence of isoleucine; by the use of ^{14}C-labelled valine they were able to show that the cell grew in the presence of isoleucine by utilisation of endogenously produced valine.

Similar findings are reported by Britten, Roberts & French (13) who showed a rapid and specific concentration of proline and methionine in the trichloracetic acid-soluble portion of *E. coli.* Evidence was presented that this acid-soluble material formed an intermediate stage between uptake of amino acid and its incorporation into protein, but since this group of workers start with the assumption that *E. coli* is freely permeable to amino acids, they postulate that the acid-soluble material is adsorbed on sites within the cell prior to incorporation into cellular protein.

The present findings appear to contradict the early studies of Taylor (14) who detected free amino acids in Gram-positive bacteria only. Taylor used an enzymic method of estimating internal amino acids and carried out estimations after rigorous washing of the cells. The probable explanation of the discrepancy is (*a*) Gram-positive cells in general are able to effect a higher absolute internal concentration of amino acids such as glutamic acid than are Gram-negative species; and (*b*) the internal amino acids are washed out more readily from Gram-negative species than from Gram-positive. Cohen & Rickenberg (8) carry out estimations of internal valine, etc., after efficient centrifugation from valine-containing solutions and have shown by the use

of controls in the presence of 2,4-dinitrophenol that the amount of external valine carried down in the cell pellet is insignificant in comparison with that within the cells; further washing of the cells or suspension in more dilute amino acid solution results in re-equilibration of the internal and external amino acid. Staphylococci and streptococci, on the other hand, can be washed in water or saline and even incubated in water for considerable periods without any significant loss of internal glutamic acid (14, 15). The difference between Gram-positive and negative bacteria would thus appear to reside, not in the ability to concentrate amino acids within the cells, but in the properties and reversibility of the concentrating mechanism involved.

A useful study of the distribution of amino acids in the "pool" of a number of strains of yeast, together with factors affecting depletion and replenishment, has been made by Halvorson *et al.* (16).

Activation of Amino Acids

The formation of peptide bonds between amino acids would seem to necessitate prior activation of the amino group or the carboxyl group. Enzyme systems have been demonstrated in animal tissues (17, 18, 19), in bacteria (20, 21, 22), and in yeast (23, 24) which may be involved in the primary activation of amino acids and hence in protein synthesis. The systems catalyse amino acid-dependent "exchange" reactions between ^{32}P-labelled pyrophosphate (P*—P*) and adenosinetriphosphate (ATP or A—R—P—P—P):

$$\text{A—R—P—P—P} + \text{P*—P*} \xrightarrow{\text{amino acid}} \text{A—R—P—P*—P*} + \text{P—P}$$

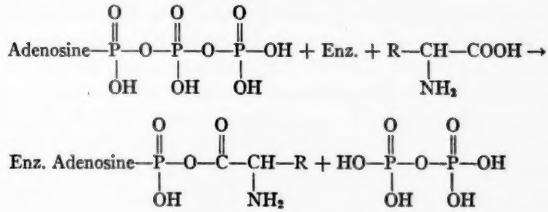
There is no net breakdown of ATP even in the presence of inorganic pyrophosphatase but addition of a high concentration (molar) of hydroxylamine depresses the exchange and results in the formation of amino acyl hydroxamates and either inorganic pyrophosphate or orthophosphate depending on conditions:

$$\text{Amino acid} + \text{ATP} + \text{NH}_2\text{OH} \rightarrow \text{Amino acyl-NHOH} + \text{AMP} + \text{P—P}$$

Similar reactions occur in the activation of acetate in yeast (23, 25, 26) and other fatty acids in animal tissues (27), with coenzyme A (CoA) replacing hydroxylamine:

$$\text{Fatty acid} + \text{ATP} + \text{CoA} \rightleftharpoons \text{Fatty acyl-CoA} + \text{AMP} + \text{P—P}$$

Berg (23, 25, 26) showed that synthetic acetyl-AMP was converted to ATP in the presence of P—P and to acetyl-CoA in the presence of CoA, and proposed that it was an intermediate in acetate activation. Hoagland (17) similarly postulated amino acyl-AMP as intermediate in amino acid activation, the complex being bound to the enzyme since no trace of such a compound could be demonstrated:



This would account for the amino acid-dependent exchange reaction. Further, the product might react in an artificial system with hydroxylamine to form amino acyl hydroxamate or, in a natural system, with the amino group of another amino acid to form a peptide bond. DeMoss, Genuth & Novelli (22) synthesised leucyl-AMP and showed that in the presence of P—P it was converted to ATP by a purified enzyme system which would catalyse a leucine-dependent exchange between P—P and ATP.

There is evidence that specific enzymes exist for various amino acids, but the systems, while present in a wide range of microorganisms, have so far been demonstrated to catalyse the exchange reaction in the presence of only eight amino acids: leucine, isoleucine, valine, histidine, methionine, tryptophan, tyrosine, and phenylalanine (20). The organisms examined include *Micrococcus*, *Neurospora*, *Aerobacter*, *Staphylococcus*, *Clostridium*, *Desulphovibrio*, *Proteus*, *Streptococcus*, *Serratia*, *Rhodospirillum*, *Azotobacter*, *Escherichia*, and *Saccharomyces;* all possessed activity. D-amino acids and the dipeptides L-leucylglycine and glycyl-L-leucine did not promote the exchange reaction. Norleucine, tryptozan and *o*-fluorophenylalanine neither enchanced exchange nor inhibited activation of their analogues, leucine, tryptophan, and phenylalanine, by extracts of *E. coli.* Since some amino acid analogues such as *p*-fluorophenylalanine (28) and azatryptophan (29) can apparently compete with phenylalanine and tryptophan in protein synthesis by *E. coli,* this inactivity is somewhat surprising. However, the tryptophan activating system purified from pancreas by Davie, Koningsberger & Lipmann (19) has subsequently been shown to be active on tryptazan. It is possible that some amino acids are activated by transacylation or by systems bound to particulate material—*S. aureus* extracts possess amino acid activating ability but supersonically disrupted organisms are able to incorporate any amino acid into their protein without the addition of soluble protein (30).

In 1941, Lipmann suggested that ATP might be involved in carboxyl activation of amino acids (31): these recently discovered enzyme systems may be those responsible.

Precursors Involved in Protein Synthesis

Halvorson & Spiegelman and their colleagues (16, 32, 33, 34) have studied the internal "pool" of yeast extractable by boiling water and shown that the free amino acids are precursors of inducible enzymes. No evidence was found

of peptide intermediates (32, 33). For instance, *p*-fluorophenylalanine and tryptazan inhibited formation of maltase but did not cause accumulation of peptides as would have been expected if these phenylalanine and tryptophan analogues had prevented the utilisation of the corresponding amino acids only and had not interfered with synthesis of peptides not containing these amino acids. Chromatographic examination of "pools" led to the conclusion that "the presence of any one analogue prevents the incorporation of its homologue and of all other amino acids as well" (32, 34). Halvorson & Cohen (35) have now shown that certain analogues (including *p*-fluorophenylalanine) while inhibiting enzyme synthesis allow protein formation in yeast and that the analogue becomes incorporated in protein in place of its homologue [see below (28, 36)]. This finding makes it less significant that "no components corresponding to peptides or other elements could be detected which were unique to samples derived from cells incubated in the presence of analogue" (32, 33).

Turba (37, 38) has studied protein precursors in a yeast, *Torula utilis*. Cultures were incubated with $CH_3{}^{14}COOH$ and the amount of ^{14}C released by ninhydrin from various fractions determined at intervals from 5 sec. to 3 hr. The material extractable by boiling 60 per cent ethanol was separated into acidic, neutral, and basic components by electrophoresis and then assayed before and after hydrolysis. The increase in the assay was assumed to arise as a result of hydrolysis of peptides. Acidic amino acids and peptides became labelled more rapidly than protein and, from time course studies, it was argued that the peptides might be precursors of protein. About 40 different peptides were separated by paper electrophoresis and chromatography of the hot alcohol-soluble material and most were found to contain glutamic acid. $^{35}SO_4{}^{--}$ also appeared more rapidly in acid peptides than in neutral or basic ones and it was found that glutathione had a "half life" of about 22 min. This is the first detailed account of the existence of large numbers of peptides in a microorganism and it will be interesting to see how general is such an occurrence. McQuillen & Roberts (39) found that 75 per cent ethanol extracted "protein" from *E. coli* after the cells had been treated with cold 5 per cent trichloracetic acid (TCA). It was later found by Roberts *et al.* (40) that ^{35}S was incorporated in alcohol-soluble and alcohol-insoluble protein at rates equal to the growth rate of the cells. However, on sulphur starvation, there was a transfer of sulphur first from glutathione and later from alcohol-soluble protein to residual protein. The same workers also found that sulphur from exogenous methionine appeared in the cold-TCA-soluble fraction of *T. utilis* in free amino acids and also in glutathione and other compounds which on hydrolysis yielded cystine and methionine. An appreciable part of the residual sulphur could be extracted by hot TCA, i.e., it was associated with the nucleic acid fraction. Snell, Radin & Ikawa (41) found that hot TCA extracted D-alanine from *Streptococcus faecalis*. The hot-alcohol-soluble peptides described by Turba (37) contained from 6 to 12 different amino acids (not always in equimolecular proportions) and would probably be

insoluble in cold 5 per cent TCA. Perhaps the different extraction procedures are responsible to some extent for divergences of opinion as to whether or not peptides occur in microorganisms.

Britten, Roberts & French (13) are also applying techniques involving radioactive tracers of very high specific activity for short-term experiments designed to yield information on the sequence of intermediates of protein synthesis. As described above, *E. coli* accomplishes a concentration of amino acids (7, 8) and Britten (42) found that the internal to external ratio was about 500 for concentrations of proline in the growth medium of up to 5 μg/ml. This intracellular proline was not displaced by washing the cells with media isotonic with the growth medium but was wholly washed out by distilled water. The pool could be completely replenished in 1 to 2 min. on incubation of the cells in growth medium containing proline. The kinetics of transfer of exogenous amino acid (proline or methionine) to pool and to protein led Britten *et al.* (13) to postulate that "pool" amino acids are precursors of protein.

Cowie & Walton (43) investigated the kinetics of formation and utilisation of "pools" of amino acids and phosphorus compounds in *Torula utilis* using ^{14}C-fructose and $^{32}PO_4^{---}$. The "pool" constituents are used for synthesis of stable end-products (proteins, nucleic acids, etc.), and the kinetic data were shown to be amenable to expression in simple theoretical mathematical form. It was concluded that "pool" amino acids exist not in the free state, but bound at sites which might be related to the sites they occupy during protein synthesis. Calculation showed that the number of "pool" amino acid molecules was three times as great as the number of nucleotide residues in the RNA so that the latter could not be the sole sites of adsorption. The difficulty of finding sufficient specific adsorption sites has not been resolved. Labelled amino acids present in the "pool" of yeast passed on into protein at a rate which was unaffected by addition of exogenous unlabelled amino acids so that such "competition" (40) as occurs between exogenous and endogenous amino acids must take place before, or at the time of, entry into the "pool." Displacement (*déplacement compétitif*) of "pool" amino acids by their exogenous counterparts such as that found in *E. coli* by Cohen & Rickenberg (8) did not apparently occur in *T. utilis* [Cowie & Walton (43)]. Neither in *T. utilis* (43) nor in *E. coli* (13) was evidence found for peptide intermediates of protein synthesis although they were looked for specifically.

Many investigators have concerned themselves with the nature of the precursors which are converted into active enzyme molecules, and Spiegelman *et al.* (34) have formulated the possibilities as follows:

Complex precursors → Active enzyme
Complex precursors + free amino acids → Active enzyme
Free amino acids → Active enzyme

Most studies deal with the formation of inducible enzymes and here the con-

clusion is that the third formulation is the correct one. In independent and complementary investigations with *E. coli*, Rotman & Spiegelman (44) using ^{14}C and Hogness, Cohn & Monod (45) using ^{35}S showed that induced β-galactosidase formed by labelled cells in a nonradioactive medium derived virtually no carbon or sulphur from pre-existing cellular components. Other evidence, too, is in favour of *de novo* synthesis from amino acids (34).

There seems to be general agreement that the "pool" constituents of microorganisms, amino acids and nucleotides, are transient intermediates, but the extent to which more complex substances such as proteins and nucleic acids are permanent end-products is still debated. The ^{14}C and ^{35}S work just mentioned (44, 45) also indicated that little if any breakdown of protein occurs in exponentially growing cells. Podolsky (46) found that the loss of labelled arginine from the protein of *E. coli* might be 8 per cent in 30 hr. but depended on various factors such as the nature of the medium in which the organisms were incubated. Koch & Levy (47) used media containing labelled glycine and unlabelled adenine to obtain *E. coli* labelled in the protein but not in the nucleic acid. When such organisms were allowed to grow in glucose-salts medium there was extremely little transfer of ^{14}C to the nucleic acid adenine. Since glycine is a precursor of purines, this implies that little protein was degraded to the level of amino acids. The minimum half time for degradation of protein was calculated to be 30 days compared with a doubling time of 1 hr. for the cells of *E. coli.* It was also found in these experiments that nucleic acids are not degraded to the purine stage since conversion of nucleic acid guanine to nucleic acid adenine was negligible although the exogenously supplied bases are interconvertible. This reinforces the earlier claim by Hershey (48). Cowie & Walton (43) have also established the "permanency" of protein and nucleic acid in exponentially growing *T. utilis.*

Halvorson, Fry & Schwemmin (16) demonstrated that under certain conditions of limiting energy supply "internal replenishment" of the amino acid "pool" of yeast cells can occur at the expense of labile proteins. This may be related to Turba's peptides which are claimed to be protein precursors. The question of "turnover" of protein in microorganisms and its relation to that in animal tissues has been discussed by Cohn and others (49). It seems generally accepted that in exponentially growing microorganisms there is little, if any, degradation of protein or nucleic acid to components which can mix with "pool" constituents.

Protein Synthesis

Conditions affecting inducible enzyme synthesis in intact cells.—Under controlled conditions the rate of an enzymic reaction can be taken as a measure of the amount of enzyme present. Consequently, investigation of the formation of enzymes provides a means of studying the synthesis of specific proteins. In general, the synthesis of enzymes accompanies the synthesis of cellular protein, and the synthesis of any one component within

a limited period of time may not represent any marked increase over the initial activity. In the case of inducible enzymes, however, there may be increase amounting to several hundredfold in the amount of enzyme during 1 or 2 hr. of incubation; consequently, inducible enzyme formation is being investigated intensively as a means of studying specific protein synthesis. In general, the formation of an inducible enzyme is dependent upon the following: the presence of an inducer which may be the substrate of the enzyme or a substance chemically related to that substrate (50); a source of energy; a source of all the amino acids required for building protein; other factors which will be discussed below.

Access of inducer to site of induction.—When investigations are carried out with intact cell preparations, the ability of the substrate or inducer to penetrate to the site of enzyme formation is clearly of importance. This matter has recently received detailed attention from Monod and his colleagues (7, 8, 51, 52) in the course of studies on β-galactosidase formation in *E. coli.* They distinguished between two phenotypically distinct lactose-negative mutants: *Lac*⁻ (absolute) mutants which cannot be induced to form galactosidase; and *Lac*⁻ (cryptic) mutants which can produce normal amounts of β-galactosidase when induced by high concentrations of thiomethyl-galactoside, but are insensitive to induction by lactose. The cryptic mutants were found to lack a galactoside-concentrating mechanism present in the wild type and in the *Lac*⁻ (absolute) mutant, so that the inability to utilise lactose as carbon source lies in the fact that lactose cannot penetrate through and is not concentrated by the cell membrane. Investigation of the nature of the concentrating mechanism shows that it is protein, that its formation is inducible, and that its concentrating activity is specific for galactosides.

Monod suggests that the passage of many substances into the cell may depend upon such specific concentrating systems and has proposed the name "permease" for such a system, indicating the relationship to an enzyme (52). Similar concentrating mechanisms have been indicated for the passage of citrate (53, 54, 55), α-glucosides (56), and amino acids (7, 8). Robertson & Halvorson (56) have studied the deadaptation towards maltose of yeasts when metabolising glucose in the absence of added nitrogen source and have found that there is an initial increase in α-glucosidase, followed by development of crypticity (meaning, in this case a loss of permease) towards maltose and eventual destruction of the enzyme. The adapted cells possessed a glucoside concentrating mechanism and inactivation of this mechanism during the deadaptation process explains the "development of crypticity." Again, induced α-glucosidase formation is first dependent upon development of the specific permease.

Composition of the incubation medium.—Wainwright & Nevill (57) have studied the effect of depletion of nitrogen reserves on inducible enzyme synthesis in *E. coli.* They found that, in the absence of an added N-source, the organism could synthesise nitrate reductase or tetrathionase but not β-galactosidase. The addition of ammonium salts to the medium increased

the formation of nitrate reductase to some extent, but had a marked effect on the synthesis of tetrathionase.

Although the presence of an energy source is necessary for inducible enzyme formation, it has nevertheless been known for many years that the presence of glucose exerts an inhibitory effect on the formation of some enzymes (58, 59). Davies (60, 61) has shown that glucose at concentrations greater than 0.001 per cent markedly inhibits the formation of invertase and lactase in *Saccharomyces fragilis* growing under constant conditions in a chemostat. The effect is not specific for glucose as either galactose or lactose prove inhibitory to invertase formation while sucrose is inhibitory to lactase formation.

Neidhardt & Magasanik (62) have investigated the effect of glucose on the synthesis of three inducible enzymes in *Aerobacter aerogenes*. The formation of histidase, *myo*-inositol dehydrogenase and glycerol dehydrogenase was inhibited by the presence of glucose in the growth medium; even the basal amounts formed in the absence of inducer were not produced in the presence of inducer and glucose. If glycerol was used as energy source, growth was stimulated to the same extent as with glucose, but there was no parallel inhibition of inducible enzyme formation. The inhibitory action of glucose was not relieved by supplementing the medium with amino acid mixtures, yeast extract or vitamin B_{12}; consequently it is improbable that the inhibition of enzyme formation is due to limitation of other growth substances. The possibility that inducer could not gain access to the site of synthesis was obviated by the following experiments: first, similar results were obtained with a histidine-requiring mutant of the organisms where histidine entered the cells at a rate sufficient to support growth despite inhibition of histidase formation: secondly, wild type strains were grown in histidine as sole source of carbon and energy, and then transferred to a minimal medium containing both glucose and histidine; increase in cell mass took place, histidine was taken up from the medium but no further synthesis of histidase took place, the initial amount being diluted out but not destroyed. The effect of growth of induced cells in the presence and absence of glucose but in the absence of inducer was then tested: synthesis of histidase and glycerol dehydrogenase ceased and the initial enzyme was diluted out but synthesis of *myo*-inositol dehydrogenase continued in a linear fashion in the absence of glucose. This synthesis was completely inhibited by the presence of 0.2 per cent glucose; but in the presence of 0.05 per cent glucose (enough to allow only a doubling of the cells) there was no synthesis during growth in glucose but linear production of enzyme during subsequent growth in the absence of glucose. The authors suggest that their results indicate a varying stability of the enzyme-forming mechanism for different enzymes and that glucose inhibits, but does not destroy, the functioning of the mechanism.

Glucose also inhibits the adaptation to lactose in *E. coli* and Cohn (63) has found that noninduced cells are more sensitive to inhibition by glucose than preinduced cells, which may continue to synthesise β-galactosidase at a

constant rate during 19 divisions after the addition of glucose. It was then found that synthesis of the galactoside concentrating mechanism or "permease" was also inhibited by the presence of glucose so that the internal concentration of inducer remained very small in noninduced cells growing in glucose plus inducer, but was high in preinduced cells under similar conditions. Cohn therefore suggests that glucose blocks the synthesis of the permease but not its functioning. He proposes that it is the ratio of internal inducer concentration to internal glucose concentration which determines the degree of inhibition of this synthesis and, consequently, of adaptation.

Course of inducible enzyme and protein synthesis.—Torriani (64) has found that the induced synthesis of penicillinase in *Bacillus cereus* is inhibited by exposure to ultraviolet radiation. The system exhibits different sensitivity at different stages of enzyme formation; it is most sensitive to radiation during the period immediately following the addition of penicillin as inducer. Torriani (64) suggested that radiation blocks the formation of new enzyme-synthesising centres but is without action on such centres following their formation. Similar effects of ultraviolet radiation on the induced synthesis of α-glucosidase in yeast have been reported by Halvorson & Jackson (65).

Løvtrup (66, 67) has studied the formation of β-galactosidase in *E. coli* during adaptation to lactose and has shown that enzyme synthesis begins during the lag phase and that the amount of enzyme synthesised per mg. new cells during the early logarithmic phase is greater than that in fully induced cells. The amount of synthesis is decreased by starvation, extraction with water, or the presence of glucose. By following the uptake of ^{35}S during adaptation, it was shown that β-galactosidase forms a greater proportion of the total protein synthesised during the early stages of growth than later and that the ratio of protein synthesis to nucleic acid is higher in adapting cells than in cells growing in the absence of inducer. Rickenberg & Lester (68) have also demonstrated that β-galactosidase synthesis may take place preferentially during induction and that, when formation of the enzyme is essential for growth, large amounts of enzyme (over 100 times the basal level) may be produced in the absence of any detectable increase in cell protein. After this initial preferential synthesis of induced enzyme, β-galactosidase formation then occurs at a rate proportional to that of total protein synthesis. If metabolism of the inducer is not required for growth, synthesis of the enzyme occurs at the proportional rate from the beginning. From these investigations it appears that inducible enzyme synthesis can be a discrete event occurring in a manner quantitatively different from, and separable from, general protein synthesis in the cell.

In this connection it is relevant to ask whether or not the inducible portion of a specific protein differs from the basal constitutive portion which is frequently demonstrable. Pollock and his colleagues (69, 70) have isolated and crystallised penicillinase from *B. cereus* strains 5/B and 569. They examined the specific activity, sedimentation and diffusion constants, electrophoretic mobility, and salt solubility of penicillin-induced penicillinase from

strain 569 and a constitutive penicillinase from a spontaneous mutant 569/H; there was no significant difference in the properties of the two preparations. They conclude that the similarity is consistent with the hypothesis that induced and constitutive enzymes are formed by basically the same mechanism. The constitutive penicillinase of another strain 5/B is, however, physicochemically distinct from that prepared from 569/H.

Dependence on cosynthesis of RNA.—If RNA synthesis is an essential concomitant of enzyme synthesis, inhibition of RNA formation should be accompanied by cessation of enzyme synthesis. Inhibition of RNA synthesis can be accomplished in various ways: by the use of specific inhibitors if such were known, or analogues of purine and pyrimidine bases which prevent RNA synthesis; by the presence of analogues which are incorporated into RNA to produce inactive nucleic acid (as below); or by restricting the supply of purine or pyrimidine to an organism requiring those bases. All these methods have been used to demonstrate a dependence of inducible enzyme formation on RNA synthesis.

Chantrenne & Courtois (71) showed that 2-thiouracil inhibits the synthesis of catalase by yeast. Spiegelman, Halvorson & Ben-Ishai (34) showed that 5-hydroxyuridine inhibits the synthesis of β-galactosidase in *E. coli;* 2-thiouracil and 5-hydroxyuridine appear to act by inhibition of uracil utilisation. Creaser (72) demonstrated the inhibition of β-galactosidase and catalase formation by 8-azaguanine in a strain of *S. aureus;* he also demonstrated the incorporation of 8-azaguanine into the RNA of the organism under these conditions. Pardee (73) found that the formation of β-galactosidase by a uracil-dependent strain of *E. coli* would occur only as long as a supply of uracil was available. Further, the synthesis of α-glucosidase in yeast has been shown to be dependent upon the presence of the "purine pool" within the cell (34). In all these investigations it has been found that enzyme formation ceases as soon as the supply of RNA precursor is exhausted (73) or RNA formation is stopped (34, 72) even if this occurs some time after induction and during the course of rapid enzyme formation. It has been argued therefore that the *synthesis* of RNA is essential for enzyme formation in these cases, any RNA which has been formed prior to inhibition being unable to promote further enzyme synthesis.

Chantrenne (74) has studied the inducible synthesis of catalase by yeast incubated in the presence of ^{14}C-labelled adenine or uracil. If cells are grown anaerobically and divided into two portions, one of which is shaken in air and the other maintained anaerobically, catalase activity increases markedly in the aerobic portion but not in the anaerobic control, and the increased activity is accompanied by increased incorporation of adenine or uracil into the nucleic acid. Aerobically-grown cells do not show increase in catalase under these conditions as the initial level is much greater than that in anaerobically-grown cells.

A question arises whether these findings are in any way peculiar to inducible enzyme synthesis. Creaser (72) found that, although 8-azaguanine

prevented the formation of β-galactosidase and catalase in one strain of *S. aureus*, the synthesis of catalase in another strain and of glucozymase in both strains was not sensitive to the presence of the analogue. Glucozymase is a constitutive system in both organisms while catalase is constitutive in one—where its synthesis was resistant to 8-azaguanine—and inducible in the other where its formation was prevented by the purine analogue. Creaser postulated that the RNA components concerned in enzyme synthesis may be of variable active life and that the RNA concerned in the synthesis of inducible catalase and β-galactosidase has an active life so short that continuous synthesis becomes necessary for continued enzyme formation. It will be necessary to investigate a wide range of enzymes and organisms before any final conclusion can be drawn concerning the relationship between the functional stability of the RNA component and the inducible or constitutive nature of the corresponding enzyme.

Further evidence in support of the hypothesis comes from the work of Kramer & Straub (75) on the formation of penicillinase in *B. cereus*. Cells were pretreated with ribonuclease during penicillinase induction in order to see whether enzyme formation would be reduced. The result was, on the contary, a marked stimulation of penicillinase formation. It seemed possible that this stimulation might be due to the pretreatment having made available a supply of nucleotides from which new RNA might be readily synthesised during induction. Accordingly, the effect of RNA on penicillinase formation in the absence of inducer was tested. The RNA preparations were made from strain 569/H, which produces constitutive penicillinase, and from an uninduced strain 569 (see 69, 70). The RNA preparation from the uninduced 569 strain had no effect on penicillinase formation whereas the extract from strain 569/H gave rise to rapid and marked enzyme formation. The activity in the extract was nondialysable but was destroyed by ribonuclease, and it is a reasonable deduction that it is associated with a specific RNA extractable from the constitutive penicillinase-producing strain but only transiently present in the inducible strain.

Hunter & Butler (76) claim that β-galactosidase formation in exponentially growing *Bacillus megaterium* can be promoted, in the absence of inducer, by the presence of RNA extracted from induced cells. Reiner (77) has shown that the formation of gluconokinase is inducible in *E. coli* and has made a detailed study of the conditions required for optimal enzyme synthesis. Reiner & Goodman (78) found that gluconokinase could be formed in washed noninduced cells by incubation in the absence of gluconate but in the presence of a "polynucleotide" preparation from induced cells. This polynucleotide preparation was free from protein and DNA within the limits of experimental error and had the general properties of RNA. Active preparations could not be obtained from noninduced cells and were only obtained from induced cells if the extraction was carried out in the presence of gluconate. The activity of the polynucleotide preparation was found to be partially dialysable; it was unaffected by ribonuclease treatment but was

fully dialysable after such digestion. It was then found that similar active material could be obtained by ribonuclease treatment of yeast RNA, although the activity of such preparations was considerably less on an optical density basis than of preparations from induced *E. coli.* The nature of the active material has yet to be identified but the general course of the experiments is remarkably similar to that leading to the isolation of the incorporation factors from nucleic acid preparations (101).

There is clearly considerable evidence in favour of a role for RNA in enzyme synthesis although the exact nature and specificity of the RNA component remains to be established. Pardee (79) found that purine- or pyrimidine-requiring mutants of *E. coli* could synthesise β-galactosidase in the absence of a supply of purine or pyrimidine when no external source of energy was available. Investigations with ^{32}P showed that, under these conditions, there was breakdown and resynthesis of nucleic acid, the breakdown making available a supply of bases for new RNA formation.

A number of investigations have centred on the question whether DNA synthesis is involved in inducible enzyme formation; in general the same types of approach have been utilised as for the work of RNA synthesis, but in no case has any positive evidence been obtained for such a requirement (34, 73, 80, 81, 82).

Enzyme Synthesis in Subcellular Preparations

Dependence on structure.—In an endeavour to achieve ready modification of the nucleic acid components in biological preparations, a number of attempts have been made to obtain protein synthesis in subcellular preparations of bacteria. The simplest modification of the cell that can be made is removal of the cell wall to leave the protoplast. This can be readily achieved in those cells whose walls contain a substrate for lysozyme. Thus, Weibull (83) has demonstrated the release of protoplasts of *B. megaterium* by lysozyme treatment of cells suspended in buffered sucrose solution. Such protoplasts possess most of the metabolic properties and capabilities of the intact cells: they can synthesise protein and nucleic acid, support the growth of bacteriophage, increase in size and, possibly, even produce buds (84). McQuillen (84) and Landman & Spiegelman (85) have demonstrated the inducible synthesis of β-galactosidase in protoplasts of *B. megaterium* and Wiame, Storck & Vanderwinkel (86, 87) have shown inducible formation of arabinokinase in protoplasts of *Bacillus subtilis*. In the case of *B. megaterium* and *B. cereus* Kramer & Straub (88) find that pretreatment of the intact cells with penicillin is necessary for later formation of penicillinase in the protoplasts. No enzyme formation occurs in the absence of pretreatment or if penicillin is added to the suspension after the addition of lysozyme. It appears that induction in this case can take place only in the intact cell.

The preparation of fractions degraded to a greater extent than protoplasts and still able to synthesise protein appears to be a more difficult matter. McQuillen (84) obtained inconsistent results with protoplast ghosts or

membranes obtained by osmotic lysis of *B. megaterium* protoplasts. Spiegelman, in a preliminary report (89), finds that the degree of dilution into water during osmotic shock is critical. Under optimal conditions, broken preparations (or "shockates") of protoplasts are able to synthesise protein, RNA, and DNA at considerable speed. Other preparations which have proved capable of protein synthesis have been made by mechanical rupture of intact cells. Thus, the disrupted cells used by Gale & Folkes (90) are prepared by exposing thick suspensions of *S. aureus* in buffered sucrose to the action of supersonic vibration at 25 kc./sec. and then separating the resulting material on the centrifuge. Under suitable conditions, these preparations can synthesise β-galactosidase in the presence of galactose as inducer, and also a number of constitutive enzymes. Nisman and co-workers (91, 92) obtained succinic dehydrogenase synthesis in fragments of *E. coli* by grinding log phase cells with powdered glass in the cold and in the presence of thioglycolate, phosphate, and sucrose. The disrupted staphylococcal preparations (90) consist of cells in which the external wall and membranes are torn with consequent escape of part of the cells contents; no description of the nature of the *E. coli* fragments is available.

Dependence of enzyme synthesis in subcellular preparations on nucleic acids.—A number of reports (93, 94, 95) have appeared in which treatment of protoplasts with ribonuclease has resulted in cessation of the ability to incorporate radioactive amino acids into the protein fraction. However, protoplasts are fragile structures and can be disintegrated by the action of ribonuclease (84, 95, 96) or other enzymes. Consequently, cessation of a synthetic activity after ribonuclease treatment cannot be taken as a criterion of RNA involvement in that activity unless at least the structural integrity of the protoplast is established; if possible, the lost activity should also be restored by addition of RNA. Spiegelman and his colleagues (85, 97) have investigated methods of stabilising protoplasts so that they can be treated with ribonuclease and deoxyribonuclease, and have studied in detail the effect of such treatments on β-galactosidase formation. It was found that treatment with ribonuclease led to removal of RNA with little or no loss of DNA and that drastic inhibition of enzyme formation occurred whenever the removal of RNA approached or exceeded 35 per cent of that initially present. On the other hand, treatment with deoxyribonuclease had no deleterious effect on enzyme synthesis unless removal of DNA was accompanied by RNA disappearance. DNA removal in the absence of a decrease in RNA led to an increased formation of β-galactosidase, and it appeared to be possible to remove 99 per cent of the DNA without any impairment of enzyme-forming function. The most recent report (89) indicates that a considerable proportion of the degraded DNA remains in the protoplasts treated with deoxyribonuclease. Further, experiments with broken protoplasts, initially containing less than 2 per cent of the original cellular DNA in precipitable or non-precipitable form, show that these structures can carry out rapid synthesis of DNA. Clearly the protoplast furnishes a most useful tool for investiga-

tions of this nature. Although results obtained so far confirm that RNA plays a role in protein synthesis, no conclusions can yet be drawn concerning DNA.

In disrupted staphylococcal cells Gale & Folkes (90) have investigated the synthesis of β-galactosidase, catalase, and glucozymase. In freshly disrupted preparations enzyme synthesis occurs if incubation takes place in the presence of a source of energy, a complete mixture of amino acids and, in the case of β-galactosidase, galactose as inducer. Nucleic acid can be removed from the preparation by incubation with nucleases or extraction with *M* NaCl. The extent of the removal of nucleic acid depends largely upon the time of exposure to supersonic vibration and RNA is removed more readily than DNA.

Two stages can be recognised in the removal of nucleic acid: a stage of partial removal when residual nucleic acid represents *ca.* 15 per cent of that initially present; and a second stage when all nucleic acid is removed as far as experimentally possible (residual = 5 to 8 per cent of the initial). At neither stage can the depleted cells synthesise protein efficiently but, whereas this ability can be restored by RNA or precursors thereof at the first stage, the addition of both DNA and RNA is necessary at the second stage. At the first stage of depletion synthesis of catalase and glucozymase can be restored by the addition of staphylococcal RNA to the incubation medium but no preparation of RNA, whether from induced or noninduced cells, has proved effective in restoring β-galactosidase synthesis. In the latter case restoration occurs if a mixture of purines and pyrimidines or of the four natural ribonucleotides is supplied. Incorporation of ^{14}C-uracil into the RNA fraction indicates that RNA synthesis is occurring during β-galactosidase formation. These results can be correlated with those of Creaser (72) who, working with intact cells of the same organism, found that the synthesis of β-galactosidase was prevented by 8-azaguanine whereas that of catalase and glucozymase was insensitive to the presence of the analogue. At the second stage of depletion, RNA or its precursors must be supplemented by DNA before enzyme synthesis can occur; DNA is effective in restoring β-galactosidase synthesis whether it has been extracted from induced or noninduced cells but is inactive in the absence of RNA precursors. There is a suggestion here that, just as in the case of the transforming principle involved in mannitol utilisation (98) DNA is necessary to enable the cell to produce a specific enzyme but the actual synthesis of that enzyme requires other factors including an RNA component and, in the case of an inducible enzyme, presence of an inducer. The synthesis of succinic dehydrogenase in *E. coli* fragments is also prevented by treatment with ribonuclease and is restored by a mixture of ribonucleotides but not by RNA extracted from the organism (92).

The interpretation of these experiments has been discussed in a number of recent reviews (89, 97, 99, 100, 101).

Amino acid incorporation and nucleic acids.—The subcellular preparations described above are all capable of incorporating radioactive amino acids into

the protein fraction under appropriate conditions (102). Incorporation will take place either in the presence or in the absence of other amino acids although its time course differs. Gale (101, 102) has set forth reasons why incorporation occurring when only one amino acid is present cannot be taken as a measure of protein synthesis, and has suggested that such incorporation can be accounted for, in part at least, by exchange reactions between the added amino acid and corresponding residues in preformed protein in the preparation. Quantitative considerations indicate that the amount of cellular protein in which such incorporation can occur represents only a small proportion (less than 2 per cent) of the total cell protein. Further, since removal of nucleic acid from disrupted staphylococcal cells results in decreased incorporation (90) it is probable that the process occurs only in such proteins as can react with nucleic acids. Incorporation in nucleic acid-depleted preparations can be restored by addition to the medium of staphylococcal nucleic acid, DNA restoring the activity to the initial level attained before depletion and RNA restoring activity to approximately half the initial level.

Digestion of the RNA preparation with ribonuclease does not destroy its ability to restore incorporation but the active material becomes dialysable. Attempts to fractionate the digested RNA led first to the separation of small polynucleotide fractions and specificity was displayed between the fractions and the amino acids whose incorporation they were able to restore to completion (103). Further investigation (101) led to the separation of highly active materials (incorporation factors) which were not associated with characterisable polynucleotides and each of which promoted the incorporation of a range of amino acids; the polynucleotide fractions first separated were also dissociated after treatment with mild alkali into incorporation factors and inactive polynucleotides. The nature of the incorporation factors has yet to be elucidated, as has their relation to RNA and the polynucleotide fractions obtained after digestion. More information has been obtained on the Glycine Incorporation Factor than on the other active materials: it is soluble in ethanol; stable to N HCl or N NaOH for 1 hr. at 100°C. although longer heating results in destruction; possesses no net charge as judged by its lack of ionophoretic mobility in buffers at pH 2.5 (phosphate), 3.5 (formate), 5.0 (acetate) or 9.0 (borate) but moves slowly toward the negative electrode in carbonate buffer at pH 10.6. Its ultraviolet spectrum (101) displays a marked peak at 280 mμ with a shoulder at 305 mμ in acid solution. In alkaline solution the spectrum shifts to show a frank peak at 345 mμ and a minor absorption at 235 mμ. The factor completely replaces nucleic acid for the incorporation of either glycine or aspartic acid but is only partially effective for a range of other amino acids. Similar factors, differing in R_F values in isopropanol-ammonia and in the range of amino acids for which complete replacement of nucleic acid is obtained, have been separated from RNA digests. It seems probable that there are a number of closely related substances involved in incorporation reactions.

In an attempt to throw light on the nature of the components involved

in glycine incorporation, a survey had been made of the action of a number of purine and pyrimidine analogues (104). Benzimidazole proves to be a feeble inhibitor whose action is markedly increased by substitution of CH_3 or Cl in positions 5 and 6. The inhibitory activity of 5,6-dimethylbenzimidazole is further increased some 50 times by substitution of an allyl group in position 1; but similar substitution of glucopyranosyl or 2,3-dihydroxypropyl groups abolishes the inhibitory activity. Benzimidazole derivatives with NH_2 in position 6 prove to stimulate glycine incorporation; the most effective stimulators so far tested are 6-amino-4-hydroxy-benzimidazole or the corresponding benzotriazole. The inhibitory derivatives are antagonised by the stimulatory ones. Thus, inhibition by 5,6-dimethylbenzimidazole is completely antagonised by equimolar concentrations of 6-amino-4-hydroxybenzotriazole.

Incorporation of Unnatural Amino Acids

Analogues of amino acids are known to inhibit the growth of organisms requiring the corresponding amino acids, and the tacit assumption has been made that the presence of such analogues leads to inhibition of protein synthesis. The demonstration that the presence of substances such as *p*-fluorophenylalanine and tryptazan leads to inhibition of inducible enzyme synthesis (32, 33) lends support to this assumption. However, the demonstration that purine and pyrimidine analogues can be incorporated into polynucleotide structures during nucleic acid synthesis (see below) raises the question whether amino acid analogues might be similarly incorporated into polypeptide structures during protein synthesis. Such a process might prevent the formation of enzymes, but not of "protein."

Kidder & Dewey (105) suggested that the inhibition of growth of *Tetrahymena pyriformis* by 7-azatryptophan might be due to incorporation of this nature, while Gross & Tarver (106) demonstrated the presence of ethionine in the proteins of the organism after growth in the presence of the analogue. Pardee, Shore & Prestidge (29) find that azatryptophan can replace tryptophan in the growth of a tryptophan-requiring mutant of *E. coli*, although such growth ceases after the protein, RNA, and cell count have approximately doubled. Similar results were obtained with tryptazan; but 5-methyltryptophan would not support growth. Azatryptophan was demonstrated in the protein of cells grown in its presence and the amount found was compatible with its having replaced tryptophan in the newly formed protein. Details are not yet available, but the authors state that there was no formation of a number of inducible and constitutive enzymes during growth in the presence of azatryptophan or tryptazan although there was some increase in the activity of serine deaminase and ureidosuccinic acid synthetase. The preliminary report also indicates that nonviable bacteriophage can be produced by infection of *E. coli* B with T_2, in the presence of azatryptophan and that these bacteriophage particles possess "defective" coats of protein containing at least 0.4 per cent azatryptophan.

p-Fluorophenylalanine can also be incorporated into the protein of bacteria and yeasts (28, 36, 107). Munier & Cohen (28) have shown that addition of *p*-fluorophenylalanine to the medium during growth of *E. coli* is followed by continued linear growth, and that the analogue can be demonstrated in the protein formed under these conditions. Analysis of the amino acid content showed that protein formed in the presence of *p*-fluorophenylalanine contained 23 per cent less phenylalanine and 47 per cent less tyrosine but the same amount of valine and leucine/isoleucine as "normal" protein. No incorporation of *p*-fluorophenylalanine took place in the presence of chloramphenicol. The presence of the analogue prevented adaptation to lactose, maltose, or xylose (as measured by growth and oxygen consumption); but the formation of β-galactosidase as a function of the increase of bacterial mass was unaffected. Protein synthesis accompanied by incorporation of analogue was also demonstrated in the presence of β-2 thienylalanine; β-galactosidase formation was suppressed in this case.

Nucleic Acid Synthesis

Synthesis of polynucleotides by cell-free enzyme systems.—Dramatic advances in our understanding of the mechanisms of nucleic acid synthesis have recently been made with the partial purification of enzyme systems which catalyse the synthesis of RNA and DNA *in vitro*. Grunberg-Manago, Ochoa & Ortiz (108, 109) found that dialysed extracts of *Azotobacter vinelandii* when incubated with amorphous ATP in the presence of Mg^{++} and radioactive orthophosphate gave a rapid incorporation of labelled phosphate into ATP. No such exchange occurred when crystalline ATP was used. However, various nucleoside diphosphates including ADP, promoted exchange although AMP did not. Ultimately it was found that the reaction is:

$$n\ \text{X—R—P—P} \rightleftarrows (\text{X—R—P})_n + n\ \text{P}$$

where R = ribose, P—P = pyrophosphate, P = inorganic orthophosphate and X is one or more of the bases, adenine, hypoxanthine, guanine, uracil, and cytosine (108, 109, 110, 111). The polynucleotides $(\text{X—R—P})_n$ were shown (in collaboration with L. A. Heppel) to be made up of 5′-nucleoside monophosphate units linked through 3′-phosphoribose ester bonds as in ribonucleic acid (RNA).

The bacterial extracts contain adenylic kinase (myokinase):

$$\text{ATP} + \text{AMP} \rightleftharpoons 2\text{ADP}$$

as well as the new enzyme which is called polynucleotide phosphorylase:

$$n\ \text{ADP} \rightleftharpoons (\text{AMP})_n + n\ \text{P}$$

and hence ATP+AMP, pure ADP or amorphous ATP (containing ADP as impurity) were all active in promoting ^{32}P exchange.

Fresh, acetone-dried, or lyophilised cells ground with alumina and extracted with 0.15 *M* KCl yield enzyme, which has been purified some 45 times by ammonium sulphate fractionation and elution from calcium phos-

phate. The polynucleotide phosphorylase can be assayed in two ways: (*a*) by the rate of incorporation of radioactive orthophosphate into nucleoside diphosphate—phosphate exchange assay; (*b*) by the rate of liberation of orthophosphate from nucleoside diphosphate—ADP or IDP. Usually ADP is used.

Incubation of the purified enzyme with nucleoside diphosphates singly or in combination results in liberation of 60 to 80 per cent of the acid-labile phosphate and formation of polynucleotide. GDP reacts more slowly and less completely than the other diphosphates. Polymers of AMP, IMP, GMP, UMP, and CMP were prepared, as were two mixed polymers: A—U polymer from an equimolar mixture of ADP and UDP, and A—G—U—C polymer from ADP+GDP+UDP+CDP in molar ratios 1:0.5:1:1. It is not known whether dinucleotides containing purine and pyrimidine analogues are metabolised, but since some of these can be incorporated into RNA (see below) it would be expected that polynucleotide phosphorylase would react with them if indeed it is concerned in RNA synthesis. The average chain length of the A—G—U—C polymer determined by end-group analysis was about 30, that of the AMP polymer about 230. Ultracentrifuge studies and light-scattering determinations indicate molecular weights between 70,000 and 800,000 for various synthetic polynucleotides. X-ray diffraction patterns of "single" polymers such as AMP polymer, are similar to those of A—U; diffraction patterns of A—G—U—C polymers are virtually identical with those of natural RNA.

The A—U and A—G—U—C polymers are not mixtures of "single" polymers. This conclusion is based on (*a*) rates of hydrolysis by ribonuclease; (*b*) amount of nondialysable "core" (112) after ribonuclease digestion; (*c*) hydrolysis of mixed polymers labelled from A—R—P*—P* by phosphodiesterase from rat and snake venom indicate bonds between mononucleotides containing different bases; (*d*) electrophoresis and ultracentrifuge studies show differences between A—U and A—G—U—C and mixtures of "single" polymers.

Polynucleotide phosphorylase is reversible and will, in the presence of orthophosphate and Mg^{++}, degrade polynucleotides. A—U and A—G—U—C polymers are split less readily than "single" polymers such as AMP polymer. Natural RNAs including that from *A. vinelandii* can also be phosphorylysed but, except in the case of RNA of turnip yellow mosaic virus, not as rapidly as the synthetic polymers. The nondialysable "core" left after digestion of yeast RNA with pancreatic ribonuclease was not attacked; nor was calf thymus DNA.

Polynucleotide phosphorylase activity has been demonstrated in extracts from aerobic and anaerobic, and Gram-positive and Gram-negative bacteria, e.g., *A. vinelandii, E. coli, Clostridium kluyveri, Alcaligenes faecalis, Staphylococcus aureus, Streptococcus faecalis, Streptococcus haemolyticus*, and *Micrococcus lysodeikticus* (110, 111, 113). It is also present in spinach and yeast extract (110, 111).

Beers (113) studied the enzyme from *M. lysodeikticus* and found it active on nucleoside diphosphates in order of rates: CDP > ADP > UDP ≫ GDP (no activity at $10^{-3}M$). AMP polymer appeared in electron micrographs as unbranched chains and light scattering studies indicated a molecular weight of 1,860,000 with a length of 2400 Å (assuming monodispersed material).

It is by no means clear whether polynucleotide phosphorylase is an enzyme concerned in RNA synthesis *in vivo*. The ease with which it catalyses the synthesis of "single" polymers, including the unnatural IMP polymer, and of mixed polymers such as the A—U polymer, and its ability to phosphorylyse these same polynucleotides in addition to RNAs from a variety of species, are properties difficult to reconcile with a function in highly specific RNA synthesis unless the enzyme is only part of a more complex system.

Synthetic polymer A—G—U—C was not able to combine with tobacco mosaic virus protein to form infective virus as does the homologous RNA (114) although the combination did form rods similar in appearance to virus particles (110).

It is not known whether A—G—U—C polymer has a specifically determined sequence of nucleotides. The most highly purified preparations of enzyme still contain 1 to 2.5 per cent of nucleic acid and this, together with the local concentrations of nucleoside disphosphates, could conceivably determine the character of the polynucleotide synthesised.

The developments in the field of DNA synthesis have been made by Kornberg and his colleagues (115, 116, 117). Working with sonic extracts of *E. coli* they showed that crude preparations converted thymidine via the 5′-phosphate (TP) and the triphosphate (TTP) to acid-insoluble material like DNA. Partial purification gave preparations which would not use thymidine and with which TTP was 10 times as effective as TP. TTP labelled with ^{32}P in the acid-stable phosphate or with ^{14}C-thymine, was used to show that the radioactivity which became acid-insoluble was released by treatment with crystalline deoxyribonuclease but not by ribonuclease, *N* NaOH (18 hr. at 37°C.) or 0.01 *N* perchloric acid (5 min. at 100°C.). The conversion of TTP to "DNA" at this stage required: ATP; a heat-stable fraction which contained DNA and which was regarded as a "primer"; and two enzyme fractions each of which was purified more than one hundredfold.

The three other deoxyribonucleotide triphosphates (adenine, guanine, and cytosine) were then synthesised from ^{32}P-labelled 5′-phosphates and ATP by a partially purified kinase. They were incorporated into DNA by crude extracts of *E. coli* at about the same rate as TTP. However, enzyme fractions which had been purified for TTP polymerisation were considerably less active on the other three substrates. Mixtures of two, three, or all four of the triphosphates showed additive or more than additive incorporation rates but even when all four were present the DNA primer was still essential. This primer was later fractionated into a nondialysable, deoxyribonuclease-sensitive substance indistinguishable from DNA and a dialysable, acid-soluble fraction. When tested using TTP incorporation as assay the dialys-

able fraction could be replaced by a mixture of deoxyadenylic, deoxyguanylic and deoxycytidylic acids. Ultimately it was found that there is an absolute need for all four deoxynucleoside triphosphates in the system. If these are provided, one of the two enzyme fractions and the ATP are no longer required. The earlier, more complex, systems apparently provided enzymes and precursors which could make the triphosphates not supplied.

It seems, then, that a moderately purified enzyme preparation from *E. coli* will give a net increase in DNA if incubated with all four deoxynucleotide triphosphates plus a DNA primer. Increases of 20 to 50 per cent in the acid-insoluble material (deoxyribose) which reacts with diphenylamine have been obtained; the 260 mμ absorption of the precipitable material increases correspondingly and a stoichiometric amount of inorganic pyrophosphate is released during the polymerisation. Apparently the reaction occurring is as follows:

$$n\,\text{Y—dR—P—P—P} \xrightarrow[\text{Enzyme}]{\text{DNA}} (\text{Y—dR—P})_n + n\text{P—P}$$

where dR = deoxyribose, Y represents one or other of the bases, adenine, guanine, cytosine, and thymine, all four triphosphates being involved. Neither diphosphates nor monophosphates can replace the triphosphates and if any one of the latter is omitted there is no incorporation of the others. Ribonucleotide triphosphates are also inactive.

The limited extent (20 to 50 per cent) of the increase in DNA may be due to the limited amount of deoxynucleotide triphosphates available. The source of the DNA primer can be *E. coli* itself or thymus, but not *E. coli* bacteriophage T_2 (it was noted that this latter DNA contains 5-hydroxymethyl cytosine in place of cytosine).

In the case of the polynucleotides containing deoxyribose just as in the case of those containing ribose, there is no proof that biologically active material is being made, although the stringent requirements for synthesis in the case of the deoxy-derivative are encouraging. It will be necessary to see whether the system can catalyse an increase in a specific DNA such as a transforming principle (118) when supplied with that principle as primer.

INTERRELATIONSHIPS OF PROTEIN, RNA, AND DNA

In attempting to disentangle the interrelationships between protein, RNA, and DNA, investigations have been made of the ability of microorganisms to synthesise each in the absence of increase in one or both of the others. Three main types of approach have been used: (*a*) omission of a specific growth factor (an amino acid or purine or pyrimidine) to restrict possibility of synthesis; (*b*) addition of analogues to prevent specific synthesis; and (*c*) treatment with drugs, such as chloramphenicol, which specifically abolish protein synthesis (1, 119), or mutagenic agents (x-rays, ultraviolet radiation, mustards) which can selectively affect DNA synthesis (65, 73, 80).

Interference with DNA synthesis.—A thymine-requiring strain 15_{T-} of *E. coli* has proved useful in demonstrating formation of protein and RNA in the absence of DNA synthesis (81, 120, 121, 122, 123). It is clear that these components can approximately double in amount without demonstrable increase in DNA. However, specific deprivation of thymine leads to "unbalanced growth" (120, 121) and death of *E. coli* 15_{T-}. A similar effect can be achieved in wild-type *E. coli* by supplying all the requirements for growth in the presence of sulphanilamide (amino acids, purines, and growth factors) except thymine. Such bacteria die at the rate of about 90 per cent in the time necessary for one division, despite an increase in turbidity of the culture. On the other hand, omission of an amino acid or purine or addition of 5-methyl-tryptophan prevents growth and death. The omission of both thymine and an amino acid has a similar effect. There was no increase in viable cell count in the absence of both thymine and purines but the turbidity of the culture increased by about 40 per cent indicating that some synthesis might occur in the absence of DNA and RNA synthesis; whether protein is made under these conditions is not known. Ben-Ishai & Volcani (123) using washed cells of *E. coli* 15_{T-} confirmed that this organism can make protein and RNA in the absence of DNA formation and found a quantitative relationship between the amounts synthesised. In various media (all lacking thymine) the ratio of protein increment to RNA increment was approximately constant at between 3 and 4 to 1. Moreover, a group of purine and pyrimidine analogues (5-hydroxyuridine, thiouracil, nitrouracil, 2-azaadenine) reduced both RNA and protein syntheses to the same extent leaving the incremental ratio unaltered. 2:6-Diaminopurine (450 μg./ml.) and chlorxanthine inhibited neither. Since protein synthesis could be prevented by chloramphenicol without impairing RNA formation, these results were taken as further evidence that synthesis of RNA is necessary for protein formation.

Borek (124, 125) found that a methionine-requiring mutant (W-6) of *E. coli* K 12 could double its content of RNA when deprived of methionine without increase in DNA or protein. Under conditions of methionine starvation there was no change in viable cell count for periods of up to 6 hr. (125) unlike the "thymineless death" of *E. coli* (120, 122). It was noted above that omission of an amino acid as well as thymine prevented death (122). After unbalanced accumulation of RNA, the mutant W-6 was uniquely slow in synthesising protein and inducible enzymes when subsequently supplied with methionine. Other mutants of *E. coli* (including another requiring methionine) neither accumulated RNA on starvation nor showed impaired protein synthesis on replenishment.

Pardee & Prestidge (126) using a leucineless, methionineless mutant derived from that of Borek (124) found that deprivation of methionine again allowed RNA synthesis with little concomitant DNA or protein synthesis; in the absence of leucine, there was only a small increase in RNA.

Interference with RNA synthesis.—Lack of purines or pyrimidines and

presence of analogues have been shown in many cases to impair inducible enzyme formation (see above), but few studies have been made of the effects of these conditions on protein synthesis in general. Mandel *et al.* (127) and Bolton & Mandel (128) determined the effect of 6-mercaptopurine on growth, synthesis of nucleic acids, inducible enzyme formation, and incorporation of labelled sulphur, glucose, formate, uracil, and phosphate by *E. coli* B. Growth in the presence of the analogue for 90 min. resulted in cells containing 58 to 76 per cent (mean 65 per cent) of the control amount of nucleic acid (mainly RNA) but which by spectrophotometric evidence contained little if any 6-mercaptopurine. The newly formed nucleic acid amounted to about half as much per unit increase in culture density as in controls; but protein synthesis (as measured both by ^{35}S incorporation and induced β-galactosidase formation) represented the same proportion of increase in culture density whether or not the drug was present. These results show that the incremental ratio of protein to RNA is not the same in control and 6-mercaptopurine-inhibited *E. coli*, a finding which contrasts with that of Ben-Ishai & Volcani (123) using other analogues (see above). It may be significant that the latter experiments were carried out on thymine-deficient cells in which DNA synthesis could not occur.

The positive correlation often found between the RNA content of cells and the rate of protein synthesis in these cells [e.g. (129, 130, 131)] is often taken as an indication that RNA plays some role in protein synthesis. It is, however, still impossible to say whether or not RNA content or the extent of RNA synthesis is directly and quantitatively related to protein synthesis. Little is known of the extent of RNA turnover rates, particularly in systems where protein synthesis occurs in the apparent absence of increase in RNA. However, Volkin & Astrachan (132, 133) found rapid turnover of RNA in *E. coli* infected with bacteriophage T2r^{+}, a system in which previously it had been thought that the formation of viral protein and DNA was divorced from RNA synthesis.

The discovery that amino acids can be activated by conversion to substituted phosphoryl derivatives (AMP-derivatives) (17 to 23) adds point to the suggestion that concomitant synthesis of protein and RNA may be a normal process. However, the relative amounts of protein and RNA in bacteria [15 amino acid residues to 1 nucleic acid base in *E. coli* according to Pardee & Prestidge (126); about eight to one according to Roberts *et al.* (40)] are inconsistent with this notion unless some RNA subsequently breaks down. Even in the ribonucleoprotein particles (40 S particles) found by Schachman *et al.* (134) in all bacterial species examined, the ratio is only four to one.

Since at present there are no means available for fractionating RNA into functionally distinct components (if such exist) it is not possible to say with certainty if RNA synthesised under abnormal conditions, e.g., in the presence of purine or pyrimidine analogues, is wholly or partly "false." This would be expected to be the case if RNA acts as a specific template and if

unnatural bases can become incorporated. A change from exponential to linear growth would be consistent with inhibition of synthesis of biologically active RNA and continued functioning of existing RNA. Unfortunately, there is no known specific inhibitor of RNA synthesis apart from analogues which might interfere with nucleotide function as well as nucleic acid synthesis. McQuillen (84, 135) found that UO_2Cl_2 appears to reduce conversion of glycine to nucleic acid purines in protoplasts of *B. megaterium* but not in the intact cells. In neither cells nor protoplasts was incorporation of glycine in protein affected by this reagent.

Interference with protein synthesis.—Chloramphenicol specifically interferes with protein synthesis in many bacterial species (1, 119) and omission of an essential amino acid necessarily has the same result. It is, therefore, comparatively simple to study the formation of RNA and DNA in systems prevented from making protein. In addition, by using bacteria infected with bacteriophage, it is sometimes possible to follow synthesis of a specific kind of DNA (136 to 140).

Pardee & Prestidge (126) followed protein, RNA, and DNA contents of a variety of amino acid-exacting mutants of *E. coli* with and without their specific amino acid requirement and in the presence and absence of chloramphenicol. As found earlier by Sands & Roberts (141), amino acid deficiency leads to impaired nucleic acid synthesis, RNA being affected more than DNA. It was also confirmed that chloramphenicol can block formation of protein while allowing that of RNA, and to some extent of DNA, to continue. The RNA formed in the presence of the antibiotic had the normal ratio of the four bases. Gale & Folkes (142) found that amino acid-exacting staphylococci incubated with glucose, purines, and pyrimidines synthesised little nucleic acid. If chloramphenicol were added the amount increased about fourfold, and with chloramphenicol and amino acid there was 12 times as much nucleic acid made. Pardee & Prestidge (126), working with *E. coli* mutants, similarly found that the required amino acids had to be present for RNA synthesis even when chloramphenicol prevented detectable protein synthesis. When only small amounts of amino acids were available, more RNA synthesis took place in the presence of the drug than in its absence. It was suggested that chloramphenicol, by preventing bond formation between amino acids, allows them to function repeatedly, possibly as nucleotide carriers in RNA synthesis.

Gros & Gros (143) found that in each of 5 amino acid-requiring strains *E. coli*, RNA and DNA synthesis would take place only in the presence of the appropriate amino acid. When protein synthesis was prevented by chloramphenicol, a trace of amino acid allowed continued formation of RNA whereas in the absence of the drug only a minute amount of RNA (25 times less) was made. Similar results were obtained when cells starved of amino acid were treated with chloramphenicol 20 min. before the addition of a small amount of the amino acid. No increase of DNA was observed under these conditions. Linear and continued formation of DNA occurred, however,

if the trace of amino acid was added a few minutes before the chloramphenicol.

A similar requirement for "prior protein synthesis" as a condition for DNA synthesis was noted earlier by Hershey *et al.* (136, 137), Burton (138), and Tomizawa & Sunakawa (139) in phage-infected bacteria. In work with *E. coli* infected with T-series bacteriophages containing 5-hydroxymethyl cytosine it was shown that the formation of DNA was prevented if conditions adequate for protein synthesis were lacking during and after infection. Such a state of affairs could be achieved by addition of chloramphenicol or 5-methyltryptophan, or by omitting a required amino acid. However, if protein synthesis was allowed to take place for only a few minutes after infection of the host by the phage, and then prevented by removal of amino acid or addition of inhibitor, DNA synthesis would proceed for long periods. Moreover, the DNA formed was characteristic of the phage both chemically (i.e., contained 5-hydroxymethyl cytosine) and biologically [it could later be incorporated into infective bacteriophage (137)].

Rosenbaum *et al.* (144) claimed that phage DNA synthesis could occur in *E. coli* infected with phage T2 even in the presence of chloramphenicol but Tomizawa & Sunakawa (139) suggest that the concentration of antiobiotic used (4 μg./ml.) was inadequate to prevent protein synthesis.

A more extended study has been made by Crawford (140) using *E. coli* B infected with phages T1 to T7 and 201 S and *E. coli* 15_{T-} infected with a newly isolated phage C 1. The phages T2, T4 and T6 which contain 5-hydroxymethyl cytosine behaved as found by others (136 to 139). Addition of chloramphenicol at the time of, or during the first few minutes after, infection completely prevented DNA synthesis. Addition later had progressively less effect. Quite different results were obtained with phages T1, T3, T5, T7, 201 S, and C 1. Even when chloramphenicol was added to the nitrogen-free adsorption medium 5 min. before transfer of the infected cells to the growth medium, there was considerable synthesis of DNA during the subsequent 30 min. period in growth medium containing drug. Chloramphenicol was used at a level (20 μg./ml.) which reduced ^{14}C-leucine and $^{35}SO_4$ incorporation to 1 to 2 per cent of the control amount; hence, it seems that prior protein synthesis was not essential in this case. The outstanding difference between the T-even phages and the others tested is the occurrence of 5-hydroxymethyl cytosine in the former and cytosine in the latter. Thus, the requirements for formation of protein before new phage DNA can be made may be related to metabolism of 5-hydroxymethyl cytosine. It should be pointed out that it is not yet certain that the DNA synthesised in the presence of chloramphenicol is phage DNA; this is difficult to characterise in the case of phages whose DNA has the same bases as that of the host bacterium.

Incorporation of unnatural bases into nucleic acids.—A metabolite analogue can function in two known ways: it may act as a competitive inhibitor of an enzyme normally concerned with metabolism of the corresponding

natural substrate; or it may be metabolised in place of the natural substrate and thus become part of the product of the enzyme reaction. If it is metabolised, the unnatural product may (*a*) be inert where the natural product has some essential activity or (*b*) prove toxic to some later process in the metabolic pathway. The latter process has been called "lethal synthesis" by Peters (145). Numerous analogues of purines and pyrimidines have been shown to act as growth inhibitors for bacteria. The first indication that some of these analogues may act by a process of "lethal synthesis" was obtained by Mitchell, Skipper & Bennett (146) when studying the action of 8-azaguanine. Matthews (147, 148) showed that 8-azaguanine becomes incorporated into the RNA of tobacco mosaic virus and can be isolated therefrom as the corresponding nucleotide; the incorporation is accompanied by loss of infectivity. Addition of 8-azaguanine to the growth medium is followed by incorporation of the analogue into the nucleic acid of a variety of bacteria (72, 149) but, except in the case of *B. cereus*, it is incorporated into the RNA fraction only. Mandel, Sugarman & Apter (150) have studied the distribution of ^{14}C-labelled 8-azaguanine in the nucleic acid of *B. cereus* and have found that the radioactivity is present almost exclusively as 8-azaguanylic acid in the RNA, less than 5 per cent of the radioactivity being associated with the DNA fraction.

It is a common finding in investigations of such incorporation that, although the presence of the analogue in the growth medium brings about a degree of growth inhibition, there is little or no relation between the amount of unnatural base incorporated and the degree of growth inhibition. Mandel (151) finds that 8-azaguanine is incorporated only by actively growing *B. cereus* cells and that the amount of uptake is proportional to the growth. If 8-azaguanine is added to a growing culture, a new, slower rate of growth is established within the first 30 min. after the addition. However, incorporation of the analogue does not take place into DNA during this period while the incorporation into RNA is linear throughout growth. When the new growth rate is established, steady accumulation of azaguanine into the RNA continues with no further effect on the rate of growth. If guanine is added to cells which have already taken up 8-azaguanine, then the growth inhibition is reversed and some of the analogue is removed from the RNA; the reversal nevertheless leaves a large amount of 8-azaguanine still in the cellular RNA and this has no effect on the growth. Mandel concludes that not all the RNA can be concerned in cell proliferation. He puts forward two possible explanations of the effects obtained. One, that the addition of 8-azaguanine is followed by the immediate incorporation into a particular part of the RNA which affects the rate of growth and, two, that the material which accumulates during the consequent growth has a different function or, alternatively, that the presence of 8-azaguanine antagonises the action of some cofactor which is required for growth but not necessarily involved in RNA synthesis.

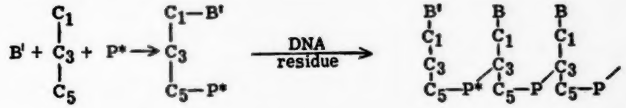
Since the initial demonstration that 8-azaguanine becomes incorporated into nucleic acid during growth inhibition, the action of a number of other

purine or pyrimidine analogues have been examined from this point of view. Koch (152) could find no appreciable incorporation of methyl-substituted xanthines into the nucleic acids of *E. coli* but 2-thiouracil has been shown to be incorporated into the RNA of tobacco mosaic virus (153, 154) and Dunn (155) has found that 5-chloro-uracil is incorporated into both the RNA and DNA of *E. coli* growing in its presence. A number of investigations have been made of the 5-halogen-uracil derivatives. Weygand, Wacker & Dellweg (156) showed that 5-bromo-uracil labelled with ^{82}Br becomes incorporated into the nucleic acid of *S. faecalis* growing in its presence and that resting cells released labelled bromo-uracil on further incubation in the presence of thymine. Later work by Dunn *et al.* (157) confirmed that 5-chloro-, 5-bromo- and 5-iodo-uracil are inhibitors of the growth of *E. coli* and that the effects of the bromo- and iodo-derivatives are antagonised by thymine but not by uracil. The bromo- and iodo-analogues were found solely in the DNA fraction of the organisms after incorporation. 5-Bromouracil was also detected in the DNA of bacteriophages T_2r and T_5 growing in *E. coli* in medium containing the analogue. Substances with the characteristics of 5-bromouracil-deoxyribonucleotide and 5-bromouracil-deoxyriboside were isolated from the DNA of *E. coli* or T_5 bacteriophage after hydrolysis with deoxyribonuclease, followed by appropriate treatment with rattlesnake venom diesterase and 5′-nucleotidase. Quantitative evidence was obtained indicating that the halogenated uracils (other than the chloro-derivative) replaced thymine residues in DNA. The extent of the replacement can be correlated with the similarity of the size of the halogen substituent to the size of the methyl group (158). *E. coli* containing 5-bromouracil in its DNA is still capable of division and contains the usual total amount of DNA; growth of such organisms may give rise to unusual morphological forms but need not be accompanied by phenotypic changes or typical mutations (159). Attempts have been made by Zamenhof *et al.* (158) to obtain incorporation of 5-bromouracil into the DNA of nondividing cells. Cells were first grown in a broth medium for 7 hr. and then transferred either to a second broth medium containing 5-bromouracil or to a salt solution containing the analogue with and without glucose. No significant incorporation was obtained during the second incubation in salt and analogue whether the first period of active growth took place in the presence or absence of analogue; however, when growth took place in the presence of 5-bromouracil and the cells were then incubated in broth containing 5-bromouracil for a second period of 17 hr., further incorporation took place although there was no increase in the cell count; the authors conclude from these results that incorporation does take place in nondividing cells although positive results were only obtained when prolonged incubation took place in a growth medium.

Cohen & Barner (122, 160) have shown that growth of *E. coli* under conditions of thymine deficiency is a lethal process, 90 per cent of the cells becoming nonviable after one division. If thymine deficiency is supplemented by deficiency of an amino acid or purine, the death of the cells is

markedly reduced. A number of thymine analogues were tested for their ability to compete with thymine for the growth of the thymine-less strain 15T-. Both uracil and 5-bromouracil were found to act as thymine antagonists and the incorporation of 5-bromouracil into DNA was followed by death of the cells after one division. 5-Bromouracil was relatively ineffective in the presence of thymidine whereas thymine-ribofuranoside, spongothymidine, and 5-bromouracil-deoxyriboside were inhibitors of growth unaffected by the presence of thymidine; these latter compounds did not inhibit nonexacting strains of *E. coli*. Dunn & Smith (161) have shown the presence of a new base in the DNA fraction of *E. coli* 15T- grown either under conditions of thymine deficiency or in the presence of 5-aminouracil or 2-thiothymine. Neither of the analogues is incorporated into the nucleic acid fraction and the new base has been identified as 6-methylaminopurine. Material with the characteristics of the corresponding nucleoside has been prepared by treatment of the DNA with deoxyribonuclease, snake venom diesterase and 5′-nucleotidase. The appearance of 6-methylaminopurine residues in the DNA fraction is accompanied by a decrease in both thymine and adenine residues.

The mechanism of incorporation of 5-bromouracil has been investigated with the thymine-requiring *E. coli* (162) by supplying the analogue and ^{32}P-orthophosphate to a culture nearing the end of the logarithmic phase in normal thymine-containing medium. Incubation was continued for a further 17 hr. during which time the cell count increased by 21 per cent. The DNA fraction was isolated and the 5-bromouracil deoxyribonucleotide separated; its specific activity was found to be comparable to that which would be expected if all its phosphorus were obtained from the medium, and greatly exceeded that of the other nucleotides. The authors suggest that, since the results demonstrate that nucleotide-P is acquired from the pool as 5-bromouracil enters the DNA, nucleotides as such can enter DNA. The course of events is pictured in the following illustration:



The abnormal base B′ and radio-phosphorus P* become attached to pentose at the 1 and 5 positions to form a 5′-nucleotide which then condenses with the 3′-hydroxyl group of another nucleotide at the end of a DNA chain.

The mode of action of 6-azathymine as a growth inhibitor has been investigated by Prusoff and co-workers (163 to 166). 6-Azathymine competitively inhibits the growth of *S. faecalis* and *Lactobacillus casei* in media containing thymine or thymidine. It is noninhibitory for *Leuconostoc citrovorum* growing in the presence of either citrovorum factor or thymidine but inhibits the growth of *Lactobacillus leichmanii* in the presence of thymidine or

pteroylglutamic acid and vitamin B_{12}. Washed suspensions or extracts of *S. faecalis* will split thymidine to thymine with dissimilation of the deoxyribose; in the presence of azathymine the deoxyribose is transferred to the analogue to form azathymidine but there is no inhibition of nucleosidase activity. 6-Azathymidine is a more potent inhibitor of the growth of *S. faecalis*, *L. leichmanii*, or *Lactobacillus acidophilus* than is azathymine so that the fixation of deoxyribose by azathymine is not the cause of its inhibitory action. Thymidine is a more effective antagonist of azathymine than thymine but the concentration of azathymidine producing 50 per cent inhibition of growth is the same whether thymine or thymidine is present as competitor. The action of either azathymine or azathymidine is only partially reversed by the subsequent addition of either thymine or thymidine. No actual demonstration of incorporation of the analogue into DNA seems to have been made although evidence is available which suggests that azathymine is incorporated into a unit in the cell more complex than the nucleoside.

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ACETIC ACID BACTERIA[1,2]

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The acetic acid bacteria, yeasts, and the lactic acid bacteria have been used from ancient times in the domestic arts of vinegar and wine production, of brewing and baking, and in the preparation of buttermilk, cheese, pickles, and the like, although the nature of the processes was hardly realised. It was Kützing's demonstration of the living nature of "mother of vinegar" which revealed, for the first time, that acetification of wine was caused by living organisms. Pasteur's studies on vinegar and wine spoilage, the early work of Hansen on the acetic acid bacteria from beer, Brown's isolation of *Acetobacter xylinum* and study of its remarkable capacity to synthesise cellulose, Bertrand's isolation of this organism from fermented mountain ash berry juice and recognition of its remarkably specific oxidative potentialities (Bertrand's Rule), and the contributions of the Delft School (particularly of Kluyver *et al.*,), all represent the classical epoch in the history of the acetic acid bacteria and are too well-known to be repeated here. Many useful and important data were supplied later by Henneberg and by Hermann and Neuschul as well.

To cover every aspect of the acetic acid bacteria from the earliest periods comprehensively, meaningfully, and usefully would take a whole monograph. The purpose of this review, therefore, is to cover only the "recent" studies on this group. The author has arbitrarily chosen, for the greater part, only publications appearing after 1940. For it was during that period that our ideas about intermediary metabolism and the role of growth factors in nutrition and metabolism grew out of infancy and this led to more meaningful experiments on the acetic acid bacteria. Passing mention of the earlier papers has been made wherever they were deemed relevant and useful; the original references can be traced through any of the earlier reviews. The

[1] The survey of literature pertaining to this review was concluded in December 1956.

[2] The following abbreviations will be used: ATP (adenosine triphosphate); CoA (Coenzyme A); DHA (dihydroxyacetone); DPN (diphosphopyridine nucleotide); DPHN (diphosphopyridine nucleotide, reduced); DPT (diphosphothiamin); E-M-P (Embden-Meyerhof-Parnas); FAD (flavin-adenine-dinucleotide); FMN (flavin mononucleotide); F-6-P (fructose-6-phosphate); F-1,6-DP (fructose-1,6-diphosphate); GLA (glyceraldehyde); G-1-P (glucose-1-phosphate); G-6-P (glucose-6-phosphate); 2-KG(A) (2-ketogluconic acid); 5-KG(A) (5-ketogluconic acid); α-KG (α-ketoglutarate); PABA (*p*-aminobenzoic acid); TCA (tricarboxylic acid); TPN (triphosphopyridine nucleotide); TPNH (triphosphopyridine nucleotide, reduced).

[3] The author is indebted to Drs. I. C. Gunsalus and J. L. Stokes for many helpful suggestions concerning the preparation of this manuscript and to the Central Drug Research Institute authorities for numerous courtesies.

omission of certain references, particularly the more recent ones, should not be construed as a reflection on their scientific value; lack of space, the author's own bias, or the inaccessibility of the papers have to bear the blame for this. The literature on the acetic acid bacteria is redundant with innumerable species names and, as far as possible, the original names have been retained to avoid confusion.

Reviews of work on special aspects of the acetic acid bacteria are available (1 to 5). An excellent general review by Vaughn (6) deals with most aspects of these organisms.

Morphology and Systematics

These aspects have not received as much attention as the others, although there are numerous literature citations. Recent studies on the formation of cellulose, capsule and pigment, and on the catalase character have yielded biochemically significant, though taxonomically confusing, results.

Capsule.—Whether *Acetobacter* form capsules has been widely debated. But it is now certain that capsule formation has been demonstrated at least in the cases of *Acetobacter turbidans*, *Acetobacter viscosum* and *Acetobacter capsulatum* (4, 5). The chemical nature of the capsule and the mechanism of its formation are discussed later. The presence of capsule was claimed not only in the above mentioned species but also in all of the species examined (4). Since the negative staining method is not without its pitfalls, the report that all species are encapsulated needs confirmation, although the authors have tried to meet part of the objection by using smears rather than cell suspensions.

Motility and flagellation.—Vaughn (7, 8) reported that *Acetobacter rancens*, *A. melanogenum*, *A. oxydans*, and also *A. aceti* were motile with polar flagella. However, Leifson's recent demonstration of peritrichous flagellation, if any, in the complete oxidizers (*A. aceti* and *A. rancens*) contradicts Vaughn's findings and has created a nice taxonomic problem. *A. suboxydans* and *A. roseum* were motile with multitrichous polar flagella while *A. xylinum* was not motile (9, 10). Another organism with pronounced motility, *A. mobile*, was isolated by Walker, *et al.* (11). Only young cultures of *A. peroxydans* were found to be motile (12). The type of flagellation was not reported in either case. A closer examination (especially electron microscopic) of a large number of strains of different species is highly desirable, because of the lack of adequate information on flagellation in this genus.

Giant colony characteristics and type of film formed have also been recommended for purposes of species classification, but their value has yet to be proved (4, 13).

Catalase content.—Another catalase-negative species, *Acetobacter paradoxum* (3), in addition to Visser't Hooft's *A. peroxydans*, has been described (3). Optimal conditions for the determination of the catalase character (especially the age and pH) have been described (4).

Cellulose formation.—This property has been regarded until now as characteristic of *A. xylinum*. The other cellulose-forming organisms, namely *A. xylinoides* and *A. acetigenum*, have been regarded as varieties of *A. xylinum* (5). The report that *A. pasteurianum* and *A. kuetzingianum* can also form cellulose (14, 15) needs confirmation. Certain recent findings cast doubt on the validity and the immutability of this characteristic (16, 17). Swirling a growing culture of *A. xylinum* (16), or the addition of arsenite (to $0.0005M$) to cultures of *A. acetigenum* (17) leads to a preponderance of cellulose-less mutants. But a more serious situation is encountered in the report of Shimwell (18) who claims to have isolated, from pure cultures of the noncellulose forming *A. mesoxydans*, mutants which form cellulose and which are indistinguishable from *A. xylinum;* some of the mutants are said to resemble *A. orleanens* and *A. rancens*. Conversely, cultures of *A. xylinum* were shown to consist predominantly (75 per cent) of noncellulosic cells.

Oxidation of ethanol to acetic acid.—All species of this genus are potent oxidisers of ethanol. However, this ability can no longer be regarded as the distinguishing characteristic of this genus. The fluorescent pseudomonads also possess this property which can, however, be demonstrated only in buffered media (19). It is to be expected that a great many organisms would possess this capacity, but a rapid and complete oxidation of ethanol to, and an accumulation of, free acetic acid in an acidic medium is characteristic of this group; most members of this group oxidise acetic acid further to CO_2 and water and this capacity for "overoxidation" has been used for species differentiation (3, 5, 7).

Ability to utilise NH_4-N.—This aspect is covered in the nutrition section. The ability to grow in Hoyer's medium (3 to 7) is of considerable diagnostic value though two species besides *A. aceti* are capable of growth in this medium (3). Certain strains of *A. aceti* were, however, reported to have lost this capacity after continued transfer in conventional maintenance media (4).

Systematics.—On the basis of flagellation and acetate oxidation, Leifson (10) has suggested that the genus be split into two: *Acetobacter*, comprising the species that oxidise acetate and have peritrichous flagella, if any; and *Acetomonas*, consisting of species which cannot oxidise acetate and are polarly flagellated. Similar grouping is also suggested by the nutritional experiments of Rainbow, *et al.* (20, 21) whose lactophilic and glycophilic groups correspond to the *Acetobacter* and the *Acetomonas*, respectively; these in turn correspond to the two physiological types of Visser't Hooft. Until much more extensive and definitive data are available, it is desirable to keep all species of the acetic acid bacteria in one genus. This group may, therefore, be defined as comprising of Gram-negative, generally catalase-positive and peritrichously or polarly flagellated rod-shaped oxidative organisms which are capable of growth in an acidic medium, and of oxidising ethanol to acetic acid.

Various schemes have been proposed for the species classification of this genus. Henneberg's classification of these bacteria into four groups based on the habitat is not tenable since the same species occurs in very different habitats; he also created a number of new species. Similarly, Walker *et al.* (4, 11, 22, 23) have described a few new species. Visser't Hooft's physiological types were developed into a system by Vaughn (7). This scheme reduced the number of species considerably, as was necessary. The more recent system of Frateur (3) is the most comprehensive one proposed until now. Catalase character, the ketogenic capacity, and the ability to oxidise acetate to CO_2 and water, have been used to assemble these organisms into four groups which are then subdivided into various species according to their ability to oxidise glucose, to utilise NH_4-N as the sole source of nitrogen in Hoyer's medium, and to form pigments and cellulose. The remarks of van Niel and Stanier are very appropriate here:

"In an attempt to subdivide the organisms belonging to one natural group of bacteria into species, one would have to create as many species as there are organisms which differ in sufficiently fundamental characteristics, regardless of the existence of the intermediate types. It depends entirely on the 'scientific tact' of the investigators to decide which character shall be deemed worthy of the designation 'sufficiently fundamental' " (24).

It is the author's opinion that Frateur's system is the best one available now. There are a number of anomalies in the results and incompatibilities in the grouping of the species as noted above and the author passes these on to taxonomic experts for judgement.

Nutrition

Until recently there was little information available on the specific nitrogen, carbon, and growth-factor requirements of the acetic acid bacteria, except in the cases of *A. aceti*, *A. lovaniens* and *A. peroxydans* (3 to 7). Most of the earlier work had been conducted using complex media containing malt extract, yeast water, beer, and the like.

Nitrogen and carbon requirements.—Hoyer had observed in 1898 that *A. aceti* could grow in an ammonium nitrogen-mineral salts medium containing ethanol and acetate. More recently it has been shown that this organism can grow in an "adequately buffered" medium with ethanol as the sole carbon source (3); however the buffer concentration is too low (1 gm. per l.) to be very effective. Two other species, *A. peroxydans* and *A. lovaniens*, also grow in the above "modified" Hoyer's medium. Although very useful, this capacity to grow in Hoyer's medium is, therefore, no longer taxonomically characteristic of *A. aceti*.

The utilisation of ammonium nitrogen is profoundly influenced by the carbon source. When glucose replaces ethanol, *A. xylinum* (25), *A. xylinoides*, *A. acetigenum*, *A. acetosum*, *A. kuetizingianum*, *A. mobile*, and *A. turbidans* (26) also proliferate in the above medium. Thus, *A. xylinum*, unlike *A. aceti*,

cannot synthesise the necessary carbon structures from ethanol. However, certain strains of *A. xylinum* also require PABA and valine, isoleucine, and alanine for growth (27, 28).

The addition of growth factors to the above glucose-mineral salts medium promotes the growth of many strains of *A. suboxydans* (25) and *A. melanogenum* (25, 29, 30). Variations among the strains are quite common. *A. suboxydans* 621, which is the most widely used strain, requires six amino acids, two being accessory (31). Some of the x-ray-induced mutants of *A. melanogenum* require proline and others serine or glycine and adenosine (30). *A. suboxydans* 621 (31), *A. oxydans* B-685, and *A. rancens* B-65 (29), which require amino acids for growth, can also utilise NH_4-N in the presence of the required nutrilites. It is rather surprising that many species cannot synthesise alanine (26). There have not been any extensive or intensive studies on the amino acid requirements of the acetic acid bacteria.

The source of carbon is of critical importance in the growth of these organisms; witness the efficacy of glucose in supporting the growth of *A. xylinum* in an NH_4-N mineral salts medium. Many strains of *A. suboxydans* and *A. melanogenum* cannot utilise lactate, pyruvate, ethanol (25), inositol (32), acetaldehyde, acetate, G-1-P, G-6-P, F-1, 6-DP, F-6-P, GLA, DHA, or TCA cycle intermediates (25, 33, 34), as a source of carbon and energy even in a medium containing all of the known growth factors, casein hydrolysate, purines, etc. (25, 32, 33). The ability of yeast autolysate, liver extract, and such other complex materials to support growth in the above media is due to their content of a utilisable carbon source (34). Indeed glucose, glycerol, sorbitol, or any carbon compound suitable for the growth of *A. suboxydans*, was capable, at 0.1 per cent level, of sparking growth in a medium containing ethanol, erythritol, or inositol as the chief carbon source (32, 33, 34). Glucose and sugar alcohols, but not lactate or the TCA cycle intermediates, are satisfactory sources of carbon for the growth of the 'glycophilic *A. viscosum* group' (*A. viscosum*, *A. turbidans*, *A. capsulatum* and *A. gluconicum*). In contrast, the 'lactophilic *A. mobile* group' (*A. suboxydans*, *A. acidum-mucosum*, *A. aceti* and *A. mobile*) show poor response to sugars and sugar alcohols but utilise NH_4-N only with lactate as carbon source (20, 21). These reports on the ability to utilise for growth lactate (3, 20, 21) and acetate after prolonged incubation (20, 21) need confirmation in view of the nutritional and metabolic disability of *A. suboxydans* (25, 33, 35) and *A. melanogenum* (25, 35).

Growth factor requirements.—Nutritional work on this group really started in 1942–43 when Peterson's group reported that *A. suboxydans* 621 required PABA, nicotinic and pantothenic acids for growth (36, 37). Most strains of *A. suboxydans* as well as other species tested conform to this pattern of growth factor requirement; *A. melanogenum* (25, 29, 30) and one strain of *A. rancens* (29) require thiamine in addition. *A. suboxydans* has been used for the microbiological assay of PABA (38). In the presence of purines, this

organism responds to much smaller amounts of PABA. The lack of effect of purines above a certain level (0.03 µg. per ml.) has been interpreted as indicating the occurrence of a rate-limiting PABA-catalysed reaction below this level in the synthesis of purines (39). *p*-Aminophenylalanine is capable of replacing PABA for the growth of PABA-requiring organisms though much higher (thousandfold) concentrations were necessary (40). *A. suboxydans* produces from PABA a metabolite with marked activity for the PABA-requiring lactic acid bacteria; it reduced the growth lag by 30 per cent and increased cell yield by 50 per cent for the latter organisms, as compared with PABA. This compound is not identical with any PABA-like or folic acid-like factors (41); further reports will be of much interest. *A. oxydans* (29), *A. xylinum* (28) and *A. mobile* (20) also require PABA for growth.

The growth-promoting activity of biotin concentrates towards *A. suboxydans* was later found to be due to the nicotinic acid contaminant (42). Biotin is required for growth by *A. ascendens*, *A. pasteurianum*, and *A. acidum-mucosum* (26).

The pantothenic acid requirement is the most extensively studied aspect of the growth factor nutrition of these organisms. Much of the work has been conducted with *A. suboxydans* 621 for the purpose of determining the structure of CoA and the reaction sequence in its synthesis. Interest in this group arose because of the reports that *A. suboxydans* is capable of utilising for its growth CoA and its cleavage products (43), certain other conjugates of pantothenate (44, 45), pantoic acid and pantolactone (46). Pantothenate is required by *A. suboxydans*, *A. melanogenum* (25, 29, 30), *A. rancens* (29), *A. ascendens*, *A. capsulatum*, *A. pasteurianum*, *A. turbidans* (26) and *A. gluconicum* (20). *A. suboxydans* and *Streptococcus hemolyticus* R69D (2) respond to pantolactone but pantoic acid is much more active and equivalent to pantothenate; β-alanine itself is inactive but increases the growth response to the lactone (46). An initial pH of 6.2, rather than a lower one, favours the response to the lactone (47). Cheldelin, King *et al.* isolated from the heart muscle an active acid-labile conjugate of pantothenate (LAC) which was twice as active as pantothenate itself. In contrast to CoA, PAC does not catalyse acetylation reactions (44, 45). CoA was reported by Novelli *et al.* to be more active than pantothenate for *A. suboxydans* and it was postulated that the PAC of Cheldelin *et al.* was perhaps one of the cleavage products of CoA (43). Novelli found that one of the fragments obtained by the action of potato pyrophosphatase was 10 to 20 times more active than pantothenate for *A. suboxydans* (48); it was first thought to be a phosphate of pantothenate, but was later shown by Baddiley, *et al.* (49) to be pantetheine-4-phosphate. From an acid hydrolysate of CoA a product was isolated which contained phosphate (one mole per mole pantothenate) but neither adenine nor ribose, and was called the "Acetobacter stimulatory factor" (ASF) (48). But none of the synthetic phosphates of pantothenate

or its cleavage products supported the growth of *A. suboxydans* (50 to 53). The claim that the removal of the mercapto-ethylamine moiety from the ASF did not affect its stimulatory activity (48) and that the PAC possibly contained glutamic acid (44) are not corroborated by the later data. Brown & Snell (54) found that pantothenyl cysteine was strongly stimulatory to *A. suboxydans*, though it was inactive for yeasts and the lactobacilli and it was presumed to be an intermediate in pantetheine synthesis from pantothenate by *A. suboxydans*. Synthetic pantothenyl-4′-phosphate has 40 per cent of the activity of the nonphosphorylated compound (55). The following order of activity was observed; CoA$\simeq$pantetheine and its 4′-phosphate > pantothenyl cysteine > pantothenic acid > pantoic acid ($\geqq$pantolactone) > pantothenic-2,4-diphosphate > pantothenic acid phosphates ($\rightarrow$0), CoA being 10 to 20 times more active than pantothenate. The —SH containing derivatives appeared to be active principally in the reduced form or on autoclaving with the medium. The "stimulatory factor" was thus presumed by Snell *et al.* and Cheldelin *et al.* to be multiple in nature (56, 57). Activity of compounds was found by Brown, *et al.* (58) to depend upon: (*a*) the ease of absorption of the compound (more dissociable compounds being more effective at lower pH); (*b*) the ease of hydrolysis of the compound, and (*c*) the ability of the organism to reduce the compound (pantethine being almost inactive while pantetheine is highly active). The —SH compounds were generally more active and the —SH groups are perhaps helpful in overcoming the permeability barriers. The importance of taking into consideration the permeability barriers is stressed while correlating the growth-promoting activity of the compounds, especially the highly ionisable ones, with their role as intermediates in the biosynthesis of CoA (58).

Most strains of *A. gluconicum* (25, 26) and a number of strains of *A. rancens*, *A. melanogenum*, and *A. suboxydans* (25) seem to be most exacting nutritionally. There is only one report of the growth of *A. gluconicum* in "simple defined" media (20). In an interesting but rather short communication, the effect of pH on nutritional requirements of *A. suboxydans* has been reported. The requirements for alanine, valine, and niacin are reported to depend upon the pH and the carbon source (59). Mustard oil (and its active components like allyl isothiocyanate) have been found to be good inhibitors of these organisms (60).

The only paper on the mineral nutrition of these organisms, by Mulder, has shown that copper is essential for the growth of *A. aceti* (61). Mineral nutrition of many industrially important microorganisms is a well studied subject and it is hoped that work on this aspect will be forthcomnig.

Metabolism

The numerous earlier studies on the metabolism of these organisms have been covered by two comprehensive reviews (1, 2). Most of these pertain to the ability to oxidise various groups of compounds, particularly sugars and

sugar alcohols. The anabolic aspects, amino acid metabolism, and details of intermediary metabolism have received until now only scant attention.

Carbohydrates.—Catabolism of glucose has been extensively studied. Many reports (1, 2) have appeared on the oxidation of glucose to gluconic, ketogluconic, glucuronic, succinic, fumaric, lactic, oxalic, and acetic acids, but few attempted to study the mechanism of these transformations.

Butlin found that young cells of *A. suboxydans*, grown on buffered maize media, produced CO_2 and presumably gluconate from glucose and had greater aldolase activity than cells grown on nonbuffered media, which did not produce any CO_2 (62, 63). Subsequently, both Butlin and Kluyver, *et al.* found that the oxidative ability (especially the ability to form CO_2 during oxidation of glucose) of *A. suboxydans* varied with age and this effect could be duplicated by addition of acid to washed cell suspensions (63, 64). Hence, it was concluded that *A. suboxydans* possessed two enzyme systems for the oxidation of glucose; one system, sensitive to acid and producing CO_2, and the more acid-stable component oxidising glucose to gluconic and perhaps ketogluconic acid (63). Likewise, Kluyver, *et al.* (64) presumed that oxidation proceeds in two steps, since there was an abrupt change in the rate (oxygen uptake) of oxidation after an uptake of one atom of oxygen per mole (64).

The oxidation of glucose to gluconate is apparently performed by the particulate fraction of the cells and is perhaps cytochrome-linked (65, 66). Many species [(*A. suboxydans* (67, 68, 69); *A. acetigenum*, *A. gluconicum*, *A. orleanense*, *A. turbidans*, *A. viscosum*, *A. xylinum*, and *A. kutzingianum* (68)] form both 2-KGA and 5-KGA; *A. dihydroxyacetonicum*, *A. kefir*, *A. orleanense*, *A. ascendens*, and *A. xylinoides* form 2-KGA (67). *A. melanogenum* was reported to form considerable amounts of another reducing acid besides 5-KGA (70). A curious situation was reported in which glucose plus $CaCO_3$ was oxidised to Ca-5-KG (37 per cent), whereas the use of Ca- or K-gluconate resulted in a 76 per cent yield of 2-KGA with only traces of 5-KGA (67). Similar yields of 2-KGA have been obtained with *Pseudomonas fluorescens* and *Serratia marcescens* (71).

The preliminary steps in the glucose oxidation by *A. melanogenum* have been worked out by Katznelson *et al.* (72). Young cells of this organism, like *A. suboxydans* (63, 64), also evolve CO_2 from glucose but only after consumption of three atoms of oxygen. Old cells, young cells treated with DNP, and cell-free extracts consume 1, 2, and 3 atoms of oxygen, at the expense of 2-KGA, gluconate, and glucose respectively. 5-KGA is not attacked by these systems; glucose, gluconate, and 2-KGA all yielded the same product. It was unstable above pH 4.5, yielding characteristic polymerisation products. Evidence from periodate oxidation, borohydride reduction, and decarboxylation of the labelled compound indicated that the intermediate was 2,5-diketogluconate (72). The enzyme systems catalysing these oxidations have not yet been studied. Foda & Vaughn (74), however, found that

A. melanogenum hydrolysed maltose to glucose, which was then oxidised to 5-KGA and not 2-KGA, and that maltose oxidation was an adaptive process. Neither of these findings was corroborated by the later work of Katznelson *et al.* (73), who also found that cell-free extracts could oxidise G-1-P and G-6-P faster than glucose, indicating that alternate pathways for oxidation of glucose operate in *A. melanogenum*. These conflicting results are hard to reconcile; nonspecificity of the chromatographic method used by Foda & Vaughn [suggested by Katznelson & Tannenbaum (73)] and strain differences may be among the causes of discrepancy. Both groups of workers seem to have discontinued this interesting and important work. Detailed investigations are well worthwhile because of the new intermediate involved and because they may reveal new mechanisms of glucose oxidation and pigment formation.

5-KGA has been reported to be further degraded via an unknown pathway to D-tartaric and oxalic acids by many *Acetobacter* species (75). Walker *et al.* (76) identified tartronic acid in glucose media which had been incubated with *A. acetigenum* for a long time. However, the possibility that this might be due to a spontaneous slow chemical process has also been pointed out by the same authors who have shown that heating Ca-2-KGA with calcium hydroxide solution in an inert atmosphere yields tartronic acid, arabinose, and ribulose (77). Evidence has been presented by Kovachevich & Wood (78) for the operation of the Entner-Doudoroff pathway in *A. suboxydans* and *A. melanogenum;* 6-PG dehydrase, and 2-keto-3-deoxy-6-phosphogluconate aldolase are present in the cell-free extracts of these organisms.

Definitive data for the occurrence of the reactions of the pentose cycle in *A. suboxydans* are available from the work of Hauge *et al.* (79, 80, 81), on glycerol oxidation. Dihydroxyacetone phosphate is produced from glycerol via two pathways in *A. suboxydans* (79, 80, 81). Formation of hexose from dihydroxyacetone phosphate and ATP, and of hexose, sedoheptulose, and triose phosphate from ribose-5-phosphate, together with the presence of dehydroxyacetone kinase, triosephosphate isomerase, aldolase, phosphoglucoisomerase, TPN-specific dehydrogenases for G-6-P and 6-P-G, indicate the occurrence of the pentose cycle in *A. suboxydans* (79, 80, 81). The inability of the cell-free extracts to dehydrogenate glyceraldehyde-3-phosphate in the presence of DPN (but without added Mg ions) was taken to imply the absence of the classical glyceraldehyde-3-phosphate dehydrogenase. Also the absence of the formation of appreciable amounts of acetate from glycerol would argue against the normal E-M-P pathway (81). In later experiments Kitos *et al.* (82) have obtained indirect evidence from tracer studies for the operation of the pentose cycle and possibly the E-M-P route for the oxidation of glucose by *A. suboxydans;* details are not yet available. It thus appears that at least two pathways for the oxidation of glucose are operative in *A. suboxydans*. Further investigations on the degradation of 5-KGA, reported to be degraded to tartrate and oxalate (75), and of 2,5-diketogluconate may

reveal other pathways. The number and quantitative significance of the alternate pathways is yet to be determined.

Pentose metabolism has been studied only cursorily. The early report of Fred *et al.* (83) that aged cells of *A. xylinum* produced ethanol and acetone from arabinose whereas young cells produced CO_2 in addition, merits further work. L-arabinose and D-xylose, but not L-xylose, were found by Bernhauer *et al.* (84) to be rapidly oxidised to the respective hexonic acids by both *A. melanogenum* and *A. suboxydans* var. *muciparum;* D-arabinose was also decomposed to a considerable extent by *A. melanogenum.* In no case could the corresponding ketonic acids be detected (84). Contrarily, it has been reported that *A. suboxydans* oxidised D-arabonic acid, α-D-glucoheptonic acid (85) and D-galactonic acid (86) to the respective ketonic acids. Formation of glycolaldehyde from D-arabinose and D-xylose by *A. acetigenum* has been reported by Walker *et al.* (87). Since the experimental details are not available, it is to be hoped that the observation is not due to an artifact. Unfortunately, this interesting report has not been followed up; it would perhaps have thrown some light on the mechanism of release of glycolaldehyde from the C_2-DPT complex subsequent to the transketolase reaction. Heptitols and heptoses are oxidised by *Acetobacter* species (88).

Oxidation of ethanol, pyruvate, and acetate.—Lutwak-Mann's early observation (89) that the alcohol dehydrogenase of *A. suboxydans* is DPN-dependent has been confirmed (90, 91). The enzyme has been purified thirtyfold and shown to be DPN-linked (90). It is also present in *A. aceti* (35, 91). Both DPN and TPN are reduced by acetaldehyde in presence of *A. aceti* extracts in which transhydrogenase could not be detected (35); whether this is due to a single enzyme is not clear. A TPN-acetaldehyde dehydrogenase is also present in *A. suboxydans* (35, 91, 92). It has been purified and found to reduce DPN also; however the activity towards DPN is lost (along with much of the activity towards TPN also) by zinc-alcohol fractionation (92). TPN-specific dehydrogenases for ethanol and acetaldehyde are present in *A. peroxydans;* the aldehyde enzyme is arsenate-sensitive (12); DPN-linked ethanol dehydrogenase also has been reported in this organism (92a).

The pyruvate metabolism of *A. suboxydans* has been compared with that of *A. aceti* (91). Definitive evidence has been presented to show that pyruvate is first decarboxylated to acetaldehyde which is then oxidised to acetate by *A. suboxydans* (91, 93). The presence of pyruvate carboxylase had been reported in 1931 by Simon (94): the enzyme has been isolated (93) and considerably pruified ($Q_{CO_2}=70{,}000$) (35, 91). Comparative studies (35, 91) of carboxylases from *A. suboxydans* and from *Pseudomonas lindneri* with those from yeast and wheat germ have shown that all are qualitatively similar with minor quantitative differences [(35, 91, 95, 96) Juni, personal communication]. *P. lindneri* is the richest source of pyruvic carboxylase among these four, being nearly four times richer than yeast (35, 91). In contrast, *A. aceti*

lacks the yeast-type carboxylase and produces acetate as an intermediate from pyruvate; free acetaldehyde is not formed, nor is oxidation lipoic acid-dependent as shown by its insensitivity to arsenite (35, 91). It is perhaps similar to the lipoic acid-independent acetate-generating system of *Proteus vulgaris* (97, 98). Cell-free extracts of both *A. aceti* and *A. suboxydans* contain lipoic acid. This substance mediates in α-KG oxidation in *A. aceti*, but its function in *A. suboxydans* is still unknown (35). Pyruvate and lactate are oxidatively decarboxylated, presumably to acetate, by cell-free extracts of *A. peroxydans* in presence of TPN. In the presence of oxygen, and DPN or TPN, formate is oxidised to CO_2 and water (12, 99). Hydrogen metabolism of *A. peroxydans* and the enzymes involved therein have been studied (92a, 99).

Acetate, pyruvate, succinate, malate, and fumarate are oxidised to CO_2 and water by *A. aceti* (35, 100), *A. pasteurianum* (101, 102), *A. turbidans* (103), *A. ascendens* (100, 102), and *A. peroxydans* (92a). The Q_{O_2} values with citric and aconitic acids were very low (35, 100, 101). Arsenite strongly inhibited the oxidation of acetate, malate, and fumarate, but malonate did not exert any pronounced inhibition (35, 100, 103). Nonproliferating cells of *A. turbidans* and *A. mobile* oxidise these dicarboxylic acids, acetate, and pyruvate to CO_2 and water. The oxidation of ethanol to the stage of acetate is rapid and then slows down. The lag in the oxidation of acetate, caused by aging the cells, is partially overcome by addition of ATP, lactate, or α-KG as the energy source (103). Malate, succinate, and oxaloacetate were reported to increase the rate of acetate oxidation by *A. aceti* (104, 105); a claim was made that citrate was detected in reaction mixtures containing malate, acetate, and cells (106). The occurrence of the reactions of the TCA cycle in *A. aceti* (35) and *A. pasteurianum* (101) has been demonstrated. Resting cells of these organisms oxidise acetate and the dicarboxylic acids at similar high rates (35, 101); fresh uncentrifuged sonicates of *A. aceti* oxidise all of the TCA cycle intermediates and acetate (35). The oxidations of acetate and α-KG by cells are very sensitive to arsenite; those of acetate and succinate are much less sensitive to malonate (35). The acetate-activating system, dependent on ATP, CoA, and Mg, is present in both *A. aceti* and *A. suboxydans* (35); it could not be detected with whole cells of *A. suboxydans* (90). The condensing enzyme, aconitase, DPN- and TPN-isocitric dehydrogenases, α-KG oxidase, succinic, and TPN-malic dehydrogenases, and fumarase have all been shown to be present in the cell-free extracts of *A. aceti;* α-KG has been isolated from an arsenite-inhibited cell-extract oxidising citrate (35). Likewise, fumarase, aconitase, isocitric dehydrogenase, together with α-KG and pyruvate oxidase systems dependent on CoA and DPN, have been demonstrated in *A. pasteurianum* (101). Plausible support for the operation of the dicarboxylic acid cycle for the oxidation of acetate is provided by the work of Tannenbaum with *A. peroxydans* (12). Sonicated uncentrifuged extracts oxidised acetate, succinate, fumarate, malate, and

oxaloacetate, but not citrate, isocitrate, or α-KG. Cell-free extracts with active dehydrogenases for malate, lactate, ethanol, and acetaldehyde were inactive towards citrate and isocitrate; α-KG was not oxidised even in presence of DPN or TPN, CoA, DPT, lipoic acid, and Mn (12). Atkinson, on the other hand, has reported the oxidation of ethanol and the intermediates of the TCA cycle, but not acetate, and the presence of aconitase and TPN-isocitric dehyrogenase in *A. peroxydans* (92a). The two isocitric dehydrogenases of *A. aceti* have been separated and considerably purified (35). The TPN and the DPN enzymes from *A. aceti* resemble their counterparts from yeast (106), *Aspergillus niger* (107), and animal tissues (108). After growth on acetate, but not on glucose, *A. aceti* forms isocitritase which causes the aldolatic split of isocitrate to glyoxylate and succinate (109). In contrast to *A. aceti* and *A. pasteurianum*, *A. suboxydans* possesses only fumarase and aconitase of the TCA cycle enzymes. The concentrations are much smaller than in *A. aceti* (35), and therefore *A. suboxydans* is unable to oxidise acetate although it can incorporate acetate and ethanol into cellular material (34, 35, 82). These results provide an enzymatic basis for the taxonomic grouping of the incomplete oxidisers.

Open chain polyols.—Ever since Bertrand's discovery of the oxidative specificity of *A. xylinum*, increasing interest has been evinced in the oxidation of polyols by these bacteria. *A. suboxydans* has been extensively used in such studies, because of its ability to oxidise substrates more rapidly and completely than other species. On the basis of extensive studies, Hann, Tilden & Hudson (110) formulated a generalisation, which can be termed the "Bertrand-Hudson Rule": for the oxidation of polyols, *A. suboxydans* requires *cis*-hydroxyls with D-configuration whereas *A. xylinum* needs only *cis*-hydroxyls. A review by Fulmer & Underkofler (32) covers most of the work of the Iowa School on the oxidation of polyols. The prediction that D-talitol would be oxidised by *A. suboxydans* to tagatose (32) has received experimental support (111). *A. xylinum* produces only D-altroketoheptulose (sedoheptulose)—Bertrand's "volemulose"—from volemitol (112), whereas *A. suboxydans* oxidises volemitol more rapidly and completely to a mixture of D-altroketoheptulose and D-mannoketoheptulose (112, 113), the proportions between the two products depending upon the cultural conditions (113). A similar case is the oxidation of D-mannitol-1-C^{14} to fructose-1,6-C^{14} (114). Both ends of the molecule in volemitol and mannitol are symmetric, and hence the two products.

Similar studies have been conducted on the oxidation of the ω-deoxy sugar alcohols. Bollenbeck & Underkofler (115) were led by their studies to conclude that the specificity requirements were uncertain. However, Richtmeyer *et al.* (116) concluded that the Bertrand-Hudson Rule could still be applied by considering the $CH_3 \cdot CHOH$— group as an elongated form of CH_2OH—; in accordance with this, they found that L-fuco-4-ketose was the oxidation product of L-fucitol. It should, however, be mentioned that fac-

tors other than steric requirements may be operative. D-glucose dimethyl acetal and mercaptal possess favourable configuration but are not oxidised; nor is there any adaptation to these compounds even after several transfers (117).

Much of the work on the cell-free polyol dehydrogenases is due to Edson's group (118, 118a, 119). *A. suboxydans* possesses two systems for the oxidation of open chain polyols. One, appropriately called the "Bertrand-Hudson Enzyme," has a pH optimum of 4.5 to 5 and may be cytochrome-linked; the other is a DPN-linked system with a pH optimum of 8, producing fructose from both mannitol and sorbitol (118). The former enzyme is apparently present in the particulate fraction of the cell (sedimentable at 20,000 *g* for 2 hrs.) (66, 118) although contrary reports exist (65). The latter enzyme resembles the adaptive enzyme of *Pseudomonas fluorescens*, which oxidises both mannitol and sorbitol to fructose (120).

A number of species oxidise glycerol to DHA (121). The inability of a strain of *A. xylinum* to form DHA and the formation of DHA by *A. pasteurianum* (121) must be regarded as due to strain variations, although on taxonomic grounds, these results are incongruous. Cheldelin's group have greatly clarified the mechanism of DHA formation by *A. suboxydans* (122 to 124). Growing cells oxidise glycerol almost quantitatively to DHA, which is further oxidised by the resting cells (122, 123) to unidentified products (90). Washing of lyophilised cells results in a loss of activity towards DHA, which is restored by addition of ATP and DPN (123). There are apparently two pathways for the oxidation of glycerol to DHA as revealed by the use of cell-free extracts; one with an optimum at pH 6, independent of DPN and ATP and perhaps cytochrome-linked; the other with an optimum at pH 8, requiring DPN, ATP, and Mg ions. The presence of glycerokinase and of a DPN-linked α-glycerophosphate dehydrogenase were demonstrated as required by the second pathway (80). Pantothenate-deficient cells have a decreased ability to oxidise DHA, sorbose, and gluconate: the activity is reported to improve on addition of CoA (124), although later experiments have not revealed any CoA-dependent step in the oxidation of glycerol (81).

Different strains of *A. suboxydans* show varying degrees of specificity towards propane-1,2-diol; some attack the D-isomer (125), and others the L-isomer (126). Meso-butane-1,2-diol is oxidised to L (+) acetoin by *A. xylinum* (127), *A. aceti* (127), and *A. suboxydans* (128). In the oxidation of glycols, the D-configuration rather than the *cis* hydroxyls seems to be important (32). Evidence has been presented for the multiplicity of the dehydrogenases (all DPN-specific) catalysing the oxidation of the glycols to the corresponding ketols (129).

The specific and restricted oxidative ability of the incomplete oxidisers has been utilised for the preparation of rare sugars and for the specific oxidation of the steroids (130). The disaccharide alcohol, 1-galactosyl mannitol, is oxidised by *A. suboxydans* to a ketodisaccharide (131). Other sugars which

have been similarly prepared include: D-fructomethylose from L-rhamnitol (132); tagatose from D-talitol (111); levulose from mannitol (133); L-erythrulose from mesoerythritol (134); perseulose from perseitol (135); and acetol from propane-1, 6-diol (136).

Oxidation of cyclic polyols.—When grown in a medium containing meso-inositol and glucose, mannitol or glycerol *A. suboxydans* was reported by Underkofler *et al.* (137, 138) to produce diketoinositol. Subsequent investigations by others (139, 140, 141) have not confirmed this observation. The cause of the discrepancy is not clear. D- and L-inositol are oxidised by *A. suboxydans* to a diketo-compound, while meso-inositol yields a mono-keto-compound (139, 140), later identified as scyllo-meso-inosose (141). D-inosose, obtained by the incomplete oxidation of D-inositol, is further oxidised by *A. suboxydans*, to cyclohexane-1,3-*cis*-tetrol-4,5-dione (142); D-quercitol is oxidised to cyclohexane-1,5-*cis*-triol-2,3-dione (142); and L-epinositol to epi-meso-inosose (144). Studies on cyclohexane-1,2,3-triols led Posternak *et al.* (145) to the first generalisation that *cis*-vicinal hydroxyls are necessary for the oxidation.

On the basis of extensive work and in conjunction with considerable physical data for the existence of the 'chair' structure for the cyclohexane molecule, Magasanik *et al.* concluded that a cyclitol must satisfy two conditions in order to be oxidised; (*a*) only polar hydroxyls, those which project above or below the puckered plane of the ring; and (*b*) the carbon atom in the *meta* position to the one with polar hydroxyl (counter clockwise if north polar and clockwise if south polar) must have an equatorial hydroxyl (143, 146). These are the minimum requirements for the oxidation of cyclohexane-hexols and -pentols. Apparently there are a number of instances where these rules do not hold, particularly for triols and tetrols, as pointed out by Posternak (147). Anderson *et al.* (148) obtained similar results and postulated the necessity of an equatorial hydroxyl at the *para* position in addition to the *meta*-equatorial hydroxyl (148). A rather puzzling finding which needs clarification is that a cell-free particulate preparation of *A. suboxydans* could oxidise the cyclitols only in the presence of heat-inactivated washed cells (65) but even this did not restore the activity towards the cyclohexane-1,2,3-*cis*-triol indicating that this compound is perhaps oxidised by a different enzyme (148).

Electron transport.—The acetic acid bacteria can oxidise substrates like ethanol very rapidly ($Q_{O_2} = 1500$ to 2000); even higher values have been reported recently (149). This had led many investigators to look for the components of the electron transport system which act with such high efficiency. CO-inhibition of the respiration of *A. pasteurianum* and its reversal by light led Tamiya & Tanaka (150) to postulate that cytochromes mediated in these oxidations. The cytochrome a_1 (α band at 589 mμ) could be seen only in very thick suspensions; it was displaced by another band at 639 mμ in presence of CO (151). The a_2 content of this organism was highly variable depending

upon the age, strain, medium etc., (152). Cytochromes a_1, a_2, and a new cytochrome a_4 were demonstrated by Chin (153) in *A. peroxydans; a_2* is presumably the auto-oxidisable component. Cell extracts of this organism have been shown by Tannenbaum (99) to possess TPN-cytochrome-*c* reductase and peroxidase activities; mammalian cytochrome-*c*, but not DPN, TPN, FAD, or FMN, can act as an electron acceptor in the hydrogenase system. Antimycin-A does not inhibit the oxidations by *A. pasteurianum* cells (154) nor by cell-free extracts of *A. peroxydans* (12): Slater's factor may hence be excluded from electron transport systems of these organisms. Chance has shown that a_1 is the respiratory enzyme of *A. pasteurianum* (155, 156). The cytochromes of *A. pasteurianum* and of *A. suboxydans* are highly active: the O_2 uptake in μm per sec/ΔOD at 400 or 440 mμ with ethanol as substrate is 186 for yeast, and 4500 for *A. pasteurianum.* The ethanol dehydrogenase is also highly active, as indicated by the differences between rates of glucose and ethanol oxidation (154, 157). In *A. suboxydans,* no evidence for any *a*-type cytochrome was obtained by Smith (154, 157); the only noticeable band was at 554 mμ. The respiration in this organism proceeds through a CO-binding protohemin-like pigment which is also the oxygen transferring enzyme (156). Cytochrome-*c* oxidase activity could not be demonstrated in any of these organisms (12, 154, 156).

Polysaccharide synthesis.—The studies of Khouvine (158, 159) and of Hibbert *et al.* (160) on the effect of the source of carbon on cellulose formation by *A. xylinum* mark a reawakening of interest in this problem. Arabitol (159) and fructose (14, 15, 160) are among the most favourable substrates for cellulose synthesis by *A. xylinum;* glycerol and mannitol have also been reported to give high yields of cellulose (15, 158, 160). Pentoses and soluble starch are poor substrates for cellulose synthesis by *A. xylinum* (160) and *A. acetigenum* (15). For *A. xylinum* the disaccharides are also not good substrates and ethylene glycol is inert (160); but these are satisfactory substrates for *A. acetigenum* (15). The reports that *A. xylinum* can grow anaerobically in the presence of suitable dyes (161) and that *A. kuetzingianum* and *A. pasteurianum* can also form cellulose (14, 15) need confirmation.

Numerous reports are available on the cellulosic nature of the membranes produced by these organisms. Chemical tests (15, 162, 163), infrared (163, 164) and x-ray (15, 162, 163, 165, 166) studies, electron micrographs (163, 167), chemical degradation to glucose (15, 162, 163), acetylation and hydrolysis in metanolic HCl (115, 162, 165), and growth of cellulose-decomposing bacteria on the membranes (168), all indicate that the membranes consist of cellulose. The usefulness of these membranes in osmometry has been demonstrated (169).

Some progress has been made in the studies on the mechanism of cellulose formation. Nonproliferating cells of *A. xylinum* form cellulose anaerobically within a few hours from mannitol, fructose, and glucose, but not from mannose, erythritol, cellobiose, ethanol, and acetate (170). Cellulose synthesis by

A. acetigenum occurs even in aerated glucose media; in such media cellobiose and two unidentified compounds have been detected (17). Melibiose has been detected in glucose-lactate media after the growth of *A. acetigenum* (171). Hestrin *et al.* (163) have studied the kinetics of glucose-C^{14} incorporation into cellulose by freeze-dried cells of *A. xylinum*. Even in appropriate media, the cells of *A. xylinum* do not contain cellulose and hence it has been concluded that a surface constituent is the terminal catalyst (163). The insoluble debris, but not the supernatant, of the ruptured cells of *A. acetigenum* synthesises cellulose (172).

Isotopic data on cellulose formation have also provided important results. With glucose-1-C^{14} as the sole carbon source, most of the activity (70 per cent) was found in position 1 of the constituent glucose units of the synthesised cellulose, the rest in C-3 and C-4 (173). Cleavage of the glucose molecule into two C_3 pieces can be inferred, since 3-C and 4-C also become labelled, but the E-M-P pathway is excluded since the 6-C does not become labelled (173). With mannitol-1-C^{14}, 84 to 96 per cent of the activity was present in 1- and 6-C and the residual activity in 3-C and 4-C (174). In the presence of ethanol, there is an increased yield of cellulose from glucose (173) or mannitol (174), but neither ethanol nor acetate is significantly incorporated into cellulose (175). With glucose-2-C^{14}, the label was found to be distributed in position other than C-2, though 60 per cent was in C-2 with only a trace in C-6 (176). Apparently, there is direct polymerisation of glucose as well as cleavage into C_3 pieces and resynthesis. The 3- and 4-C of the glucose units of cellulose formed by *A. acetigenum* from lactate-1-C^{14} were 3 to 4 times as active as 2- and 5-C; the 1- and 6-C had little activity (177), indicating the occurrence of the E-M-P pathway in this organism.

An amylose-type polysaccharide is produced in beer by *A. pasteurianum* with fructose, but not glucose, as the carbon source (3) and by *A. acidum-mucosum* in malt extract media at pH 4.5 or lower (22).

A study of the ropiness in certain beers led to the isolation of *A. capsulatum* (178), which produces ropiness only when dextrins are present. Hence it was concluded that the capsules which cause ropiness are of carbohydrate nature (179). Credit for the confirmation of this conclusion and for the unravelling of the mechanism of the synthesis of the polysaccharide (dextran) is due to Hehre *et al.* (180). They identified the polysaccharide as dextran, antigenically similar to that of *Leuconostoc mesenteroides*, and obtained active cell-free preparations capable of synthesising this substance. With purified enzyme preparations from *A. capsulatum*, amyloheptaose was the best substrate for dextran synthesis, and amylotetraose was presumably the smallest active primer. The enzyme was therefore named dextran-dextrinase. Its mode of action is comparable to that of the other enzymes transforming 1,4-links to 1,6 (181). Probably a similar mechanism exists in *A. viscosum* also. The extreme sensitivity of *A. capsulatum* to streptomycin (0.01 µg./ml. at pH 6.5) led Arnold & Hall (182) to test the sensitivity of dextran-dextrinase to this antibiotic, with negative results.

Pigment formation.—Frateur (3) has prepared and examined a number of pigment fractions from cultures of *A. melanogenum*. It would be instructive to compare these with the products of decomposition of 2,5-diketogluconate.

Amino acid metabolism.—Very little work has been conducted on this aspect. The only available report pertains to the ability of the resting cells of *A. suboxydans* to deaminate oxidatively a number of amino acids. The products of deamination have not been identified. Leucine, isoleucine, and valine, which were essential for growth, were practically unattacked (31). A study of the formation of the amino acids of the glutamic and aspartic acid families by *A. suboxydans* and *A. melanogenum* would be most interesting in view of the fact that both can grow in glucose-NH_4-N-mineral salts-growth factors medium (25, 29) and that both lack the key enzymes of the TCA cycle which is believed to be the source of the carbon skeletons of these amino acids.

Industrial

Several excellent reviews of the industrial uses of the acetic acid bacteria are available (183 to 186). Lack of space does not permit even brief consideration of recent papers (187 to 190).

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CHEMOTHERAPY OF VIRAL AND RICKETTSIAL DISEASES[1,2]

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Introduction

The general subject of chemotherapy in viral and rickettsial diseases has been reviewed recently in considerable detail by Matthews & Smith (1) and Hurst & Hull (2), as well as by the authors of this review (3 to 5). It seems needless to review again the large number of reports which have already been carefully discussed in earlier reviews. The opportunity presents itself, however, for an extensive discussion of some current concepts which bear on this subject. The present review is arranged in five parts: (*a*) Delineation of present status of prevention and treatment of virus diseases. (*b*) Discussion of the possibilities of chemoprophylaxis and chemotherapy in relation to present knowledge of the biology of virus infection. (*c*) Discussion of the principles underlying laboratory investigations on virus inhibition with special reference to inhibition of biosynthetic processes. (*d*) Discussion of recent work with benzimidazole derivatives to illustrate considerations set forth in part *c*. (*e*) Discussion of recent reports not previously reviewed, with emphasis on the experimental conditions under which virus inhibition was studied.

PRESENT STATUS OF PREVENTION AND TREATMENT OF VIRUS DISEASES

Only a few of the virus diseases of man can be prevented effectively and there is no means for the adequate treatment of any, excluding those caused by the psittacosis-lymphogranuloma group of virus-like agents (6). Thus, at present, the control of human virus diseases is commonly not feasible and most are accepted as inevitable ailments which run their course without therapeutic modification. This places virus diseases in a uniquely unsatisfactory position, for with no other category of infectious processes is management so underdeveloped and the need for it so large.

Prevention of virus diseases.—Highly effective and uniformly reliable prophylaxis is available for small-pox and yellow fever (6). The vaccines used are infective virus preparations which cause mild infections that lead to enduring immunity. Efforts have been made to develop safe and effective in-

[1] The survey of literature pertaining to this review was completed in February, 1957.

[2] The following abbreviations have been used in this chapter: DNA (deoxyribonucleic acid); RNA (ribonucleic acid).

[3] Certain of these studies were aided by a grant from the National Foundation for Infantile Paralysis.

fective virus vaccines for influenza (7), measles (8), mumps (9), rabies (10), dengue (11), and poliomyelitis (12), but none has been brought to the point of acceptable usefulness in man.

Moderately effective and useful prophylaxis is also available for influenza (13), certain of the virus encephalitides (14), rabies (15), poliomyelitis (16), and some adenovirus infections (17). The vaccines employed are inactivated virus preparations which stimulate an antibody response which, in general, does not persist for long periods. Efforts to develop inactivated virus vaccines against other virus diseases have been largely unrewarding, usually because of technical difficulties. In theory, any virus disease which naturally induces immunity against a second attack could be prevented by means of a vaccine containing the agent provided the antigenic stimulus were large enough.

Prevention by means other than vaccines is feasible for certain arthropod-borne virus diseases, e.g., yellow fever (18), dengue (19), and phlebotomus fever (20), through eradication of the insect vector. It is also feasible for some virus diseases that have relatively long incubation periods, e.g., measles (21), viral hepatitits (22), and rabies (23), through use of immune serum or gamma globulin soon after exposure.

Treatment of virus diseases.—Effective therapy is available only for diseases caused by the psittacosis-lymphogranuloma group of agents (24). These relatively large infectious agents are generally considered to be more like rickettsiae than viruses, and the chemotherapeutic substances most active against them are also active in rickettsial infections (25). None of the so-called true virus diseases is favorably affected by available antimicrobial substances. The course of the disease is not altered and recovery is neither hastened nor made more likely through their use (6).

The large number and the very high incidence of unpreventable and untreatable virus diseases, particularly those of the respiratory tract (26) and of childhood (6), as well as the frequency of and the morbidity due to certain other similar maladies, e.g., viral hepatitits, infectious mononucleosis, etc., underline the dimensions of the problem and the importance of any advance, however small, in this field.

POSSIBILITIES OF CHEMOPROPHYLAXIS AND CHEMOTHERAPY IN RELATION TO THE BIOLOGY OF VIRUS INFECTION

Virus Multiplication and Disease

There is no longer any doubt that virus disease does not develop unless virus multiplication occurs (27). This basic principle is so simple and obvious that its significance in relation to prevention and the development of effective treatment may be overlooked. The only avenues now open for achieving control of virus diseases appear to be: (*a*) prevention of infection, i.e., the initiation of multiplication; (*b*) inhibition of virus multiplication in infected cells; (*c*) modification of cell and tissue responses to multiplication; and (*e*)

acceleration of the recovery process. Because of the unique character of virus multiplication which is strikingly different from that of any other infectious agent, unusual approaches to control seem inevitable.

The evidence that virus multiplication in the infected host cell can be inhibited by the external application of certain chemical compounds is now large and convincing (1 to 5). In addition, the demonstration that inhibition of multiplication by a chemical substance can lead to modification of experimental virus disease emphasizes the validity of this approach (28). Although little more than a beginning has been made in this area there is support for the idea that continued investigation of the mechanism of multiplication and means for its inhibition may be fruitful in relation to the eventual development of effective therapy.

Areas available for chemoprophylaxis.—When prophylaxis is understood to mean prevention or modification of disease at any time prior to the appearance of illness, both the preinfection and incubation periods can be considered as intervals open to chemoprophylaxis. Chemical substances could, in theory, be utilized to affect any one of a number of separable stages leading to the development of virus disease.

In summary, the following stages may be available for chemoprophylaxis: Extracellular inactivation of infective virus particles might be achieved prior to their attachment to susceptible cells. However, with the exception of the neutralizing effect of specific antibodies on extracellular virus, no useful substances have been found and attempts in this direction have been fruitless. Prevention of attachment or adsorption of virus particles to susceptible cells seems a possibility. Attempts to accomplish this with presumed analogues of cell "receptor" materials, or by chemical or enzymatic modification of the cell surface have not provided very hopeful leads. Prevention of extension of infection or migration of virus particles among susceptible cells or tissues might also be an effective procedure. This, it appears, is the means by which vaccine-induced immunity to central nervous system infection with poliomyelitis may operate. Chemical substances have so far not been shown to be effective in preventing spread. Inhibition of the multiplication process during early cell cycles in the incubation period is a possibility that may have much merit. In certain experimental infections this has already been found to be a moderately effective procedure. In actual fact, most claims of "therapeutic" successes in the laboratory can be attributed to effects attained during the incubation period, not after manifest disease has appeared. Detailed consideration of each stage is given in later sections.

Areas available for chemotherapy.—If therapy is accepted to mean favorable alteration or modification of disease after actual illness has developed, then only the period of illness itself and the recovery period are available for chemotherapy. Chemical substances could, in theory, be used to affect various features of either interval.

In summary, the following areas may be available for chemotherapy: Inhibition of further or continuing virus multiplication may be a useful

objective. Because of the instability of the infective property of many viruses, it is probable that the relatively constant concentration of infective particles in infected tissue during the actual disease results from a near balance between inactivation and production of new particles. However, chemical substances which clearly inhibit multiplication in the infected cell have so far failed to reduce the virus concentration during the plateau period.

Modification of cell and tissue reactions to the multiplication process or to its products might be attained. Infected cells are not uniformly destroyed, in fact, some animal viruses seem to cause relatively little damage to the cells in which they multiply. If the biochemical basis for cell damage were better understood, means to counteract it might more easily be developed. The gross lesions recognizable in virus diseases are, in the main, secondary manifestations which are only in part attributable to multiplication, per se. Some modification of lesions might be obtained by substances which were not directed against multiplication of the agent.

With the exception of rabies, recovery is the rule in virus diseases of man. Although the recovery mechanism is not fully understood, it has become increasingly clear that in many virus diseases development of specific antibodies is not an essential feature. It is evident also that virus multiplication diminishes and may cease as recovery ensues. The more that can be learned about the biology and biochemistry of recovery the greater will be the chance of mimicking the phenomenon by artificial means. Detailed consideration of these areas is given in later sections.

The Biology of Virus Infection

The properties of extracellular virus particles may have little relevance to those of the virus-infected cell. With animal viruses, including those which infect man, something is known about the chemical composition and physico-chemical properties of certain virus particles separated from cells, but there is very little information about their features and potentialities after they have entered susceptible cells. It is obvious that the virus-infected cell, not the isolated virus particle, is the unit which requires most study if virus infection is to be better understood. The virus-infected cell has new and unique properties of which the most obvious is its capacity to produce more particles like that which initiated the infection. In doing this the virus-infected cell may be altered and this can result in stimulation, damage, and in some instances destruction. Such alteration is the basis upon which virus disease develops.

Extracellular virus particles.—When outside susceptible cells, viruses are, of course, readily accessible to chemical influences but because of their nearly total metabolic inertness they are almost wholly unaffected by metabolic antagonists. Despite the instability of many viruses infective for man, which may lead to an infective half-life that is, in some instances, as brief as an hour or two (29), attempts to inactivate extracellular virus *in vivo* have been largely unsuccessful. Substances that readily inactivate viruses

in vitro are usually too toxic and damaging to host tissue to be used *in vivo*.

Virus particles in transit between cells or between tissues are probably no more accessible to the effects of nontoxic chemical substances than are virus particles *in vitro*. The likelihood that it will be possible to prevent or treat virus disease by a chemical attack on extracellular virus in the host seems small indeed.

Attachment of virus particles.—Attachment or adsorption of a virus particle to a susceptible cell is apparently mediated by chemical bonds, the nature of which is beginning to be understood (30). Attachment is affected by the composition of the cell environment and may be reversible if the environment is altered promptly and sufficiently (31). Whether the intact adsorbed particle penetrates into the cell, when attachment has reached the stage of being irreversible, is still not certain. However, there is no doubt that the infecting particle disappears and cannot be found regardless of how it is sought (29, 32, 33, 34). Whether disappearance results from alteration of the virus particle and a separation of its components, as with phage (35), or is to be explained on other grounds, is not yet clear with animal viruses.

Although prevention of attachment might provide a means for chemical prophylaxis, this possibility has been explored by only a few workers. Alteration of the cell surface can apparently be obtained with the so-called receptor-destroying enzyme produced by *Vibrio cholerae* (36), and also by sodium periodate (37). A modest effect on susceptibility to infection with influenza virus in the mouse and the embryonated egg has been described after treatment with either material. In addition, infection of the allantoic membrane by influenza virus has been reported to be preventable with polysaccharides which were considered to act as analogues of the cell receptor (38). Initiation of infection can be inhibited by certain sulfonic acids and it has been suggested that these compounds may interfere with adsorption or penetration of influenza virus (39).

Latent period of multiplication.—The biosynthesis of new materials with virus specificity apparently begins soon after disappearance has occurred and is thought to occupy much or most of the so-called latent period. To conserve hypotheses, it can be postulated that the mechanism of virus multiplication has similar features in bacterial, plant, and animal cells. As is well-known, phage DNA, not the intact particle, enters the bacterium and is largely if not wholly responsible for initiating the production of new particles (35). Recent evidence indicates that plant virus RNA, separated from the particle, can also initiate virus multiplication in plant cells (40). There is strong evidence that phage nucleic acid and protein are produced separately in the infected bacterial cell and that the formation of new phage particles proceeds in a series of discrete steps; mature particles do not appear in the infected bacterium until just before lysis occurs (41). Evidence for the preliminary production of virus precursor materials and their later assembly into mature particles is less direct and less secure with animal viruses. However, studies on recombination (42, 43), the kinetics of virus reproduction

(29, 44 to 47), and electron microscope investigations on infected cells (48, 49) support the concept that animal virus multiplication is essentially similar to that of other viruses.

Certain compounds with inhibitory activity appear to exert their effects during the latent period and can be presumed to affect biosynthetic events which precede the appearance of new virus particles (50, 51). The duration of the latent period has not been precisely determined with many animal viruses although its length is known in some instances. The latent period with influenza viruses in allantoic cells is approximately 3 hr. (29); with poliomyelitis virus in monkey kidney or HeLa cells about 4 hr. (52, 53, 54); with pneumonia virus of mice in the mouse lung about 15 hr. (33); and with mumps virus in the allantoic cells about 30 hr. (55). Some animal viruses almost certainly have shorter or longer latent periods than those given.

Kinetic studies with certain benzimidazole derivatives reveal that the inhibitory effect progressively diminishes the later the compound is added during the latent period. The most potent benzimidazole derivatives are active only during the early stages of the latent period, well before any new virus particles can be found (51). Because these compounds inhibit the synthesis of RNA (56, 57, 58), it would be expected that they should inhibit an early stage of the multiplication process. Other less potent derivatives of benzimidazole have a longer period of action and inhibit not only during the latent period but also during the early part of the incremental period (57, 59). The effects of one such compound on biosynthetic mechanisms are clearly not limited to interference with RNA synthesis (57).

The remarkable effects of overloading the susceptible animal cell with either infective or noninfective particles (60, 61) of certain viruses may have a bearing on the development of effective inhibitors of multiplication. With influenza and mumps viruses, an input of 10 or more particles per cell markedly alters the dynamics of virus reproduction and almost completely prevents the production of new infective particles (29, 55, 62), although it does not prevent production of noninfective particles. If the biochemical basis for this unusual and selective inhibitory effect were known it might become feasible to mimic it with chemical compounds.

As was indicated in earlier reviews (1 to 5), certain analogues of amino acids are presumed to exert their inhibitory effects through inhibition of the synthesis of protein. Detailed kinetic studies with such compounds have been made with influenza (63) and poliomyelitis (54) viruses. It appears that, in both cases, an early stage of virus multiplication which occurs before the appearance of new particles, is inhibited. With phage T_2, it has been shown that 5-methyl tryptophan interferes with tryptophan utilization throughout the latent period (64). Chloramphenicol, which inhibits protein synthesis in bacteria, is effective in preventing the multiplication of certain phages (65). However, it has not been found to have an inhibitory effect on the multiplication of viruses in animal cells.

Incremental period of multiplication.—After new materials with virus

specificity have been produced in the virus-infected cell, it appears that new particles are formed from them. This process of maturation or assembly results in the production and emergence of infective particles identical with that which initiated the infection. Virus nucleic acid, believed to carry the genetic determinants; virus protein, thought to carry the immunological specificity, and such other materials as may compose the mature virus, are brought together into discrete physical entities that may be released from the cell and then become extracellular particles (41). With certain phages, assembly of infective particles is inhibitable by proflavine; tail-less phage heads not containing DNA are produced but cannot infect (66, 67). However, proflavine would prevent a second cycle of multiplication in previously uninfected bacteria, for the incomplete particles released from the first cycle cannot initiate infection. With influenza virus, certain amino sulfonic acids appear to inhibit the release of mature particles from the infected cell (39, 63). Compounds which clearly affect the assembly or maturation process with animal viruses have not been described.

If damage to the virus-infected cell occurs mainly during the interval when virus precursor materials are being produced, then compounds which inhibit assembly or release of new particles are unlikely to prove effective as chemotherapeutic agents. However, if the presence of mature particles in or their release from the cell also contributes to damage such compounds might have desirable effects. Unfortunately, little is known of the biochemical basis for damage to the virus-infected cell.

At present it appears that if effective chemotherapy is to be achieved through attacks on the multiplication process, it is most likely to succeed through inhibition of the production of one or more virus precursor materials. However, even with potent and selective compounds which affect metabolism leading to virus synthesis more than that necessary for the maintenance of the cell, it will be essential to get them to the virus-infected cell at an early stage of the multiplication process. Because of this requirement, such compounds are likely to be more useful in prevention of disease than in treatment.

After the latent period, when new particles appear in the infected cell, they may increase in number fairly rapidly. The rate of increase in the infected animal cell itself has not been studied directly, but some indication of it has been secured in kinetic experiments with infected animal tissues. If the input of virus particles is large enough to infect all susceptible cells almost simultaneously it is feasible to study one cycle of multiplication in a manner similar to a one-step growth experiment with phage (41). All such studies show that the number of new particles increases logarithmically for some hours (29, 34, 45, 62, 68, 69) or days, in the case of slowly multiplying viruses (55, 70), and then approaches a maximal value. The kinetics of the incremental period correspond with those of an autocatalytic process in which the rate is proportional to the amount of the product (29). When smaller inocula are used, the rate of increase is also logarithmic and cor-

responds fairly well with that seen in single-cycle experiments (29, 34, 62, 70). The kinetics of adsorption, multiplication, and release in individual cycles, as well as the distributions of the several phases, could readily explain the apparent continuousness of successive cycles.

During the period of logarithmic increase, the time to double the number of particles depends chiefly on the nature of the virus but also is affected by the type of cell in which it multiplies. Thus, the doubling time with influenza or Newcastle viruses in the allantoic membrane is about 45 min. (27, 29), but with influenza virus in the mouse lung it is 2.5 hr. (34). With pneumonia virus of mice in the mouse lung the doubling time is 8 hr. (70); with mumps virus in the allantoic membrane it is about 15 hr. (55).

With but few exceptions, inhibitory compounds have in general failed to affect virus multiplication when given during the period of logarithmic increase in single-cycle experiments. The later they are added during the incremental period in such experiments the less likely they are to give any evidence of inhibition (50). Once the latent period is completed and new virus particles have appeared, the emergence of additional particles continues and is almost, if not entirely, unaffected by compounds which are highly potent inhibitors during the latent period (51). However, when smaller inocula are used and successive cycles of multiplication occur, evidence of inhibition can be obtained during the logarithmic increase period. With pneumonia virus of mice and mumps virus, which have relatively long latent periods and long doubling times, *Klebsiella pneumoniae* polysaccharide causes inhibition during the logarithmic increase period (50). Certain benzimidazole derivatives also give some inhibition during the early part of the incremental period (59).

The discovery of substances which could markedly inhibit in single-cycle experiments, even when given after the latent period, might foward the search for effective chemotherapeutic agents.

Yield of virus particles from infected cells.—The yield of new virus particles has been measured in only a few instances. The maximal yield at the concentration plateau is of the order of 1000 particles per allantoic cell with influenza and Newcastle viruses (27, 29). Similar yields have been obtained with influenza virus in tissue culture systems (71). In contrast, the yield of mumps virus is only about 50 particles per allantoic cell (55). With poliomyelitis virus in monkey kidney cell cultures yields of about 100 to 200 particles per cell have been obtained (52, 72). Technical difficulties in counting the number of particles or the infected cells have prevented measurements of yield in many instances.

Inhibition of multiplication is measured in most cases by determination of the yield of new particles from an infected tissue under standardized conditions (73). If potent inhibitory compounds are added early enough the yield is strikingly diminished. With various benzimidazole derivatives the degree of reduction in the yield is directly related to the concentration of

the compound (74). This relation provides a means for determining with some precision the inhibitory activity of a compound (75).

Reactions of the Virus-Infected Cell

The virus-infected cell is almost certainly metabolically different from the uninfected cell. The most obvious difference is of course its capacity to produce new virus particles. Both the protein and the nucleic acid that are used in the assembly of new particles have properties different from those of the uninfected cell. It may be assumed, therefore, that the metabolism of the cell is oriented in new directions by the infecting particle and that virus multiplication is dependent on biosynthetic processes which are not identical with those of the uninfected cell.

Cell damage from infection.—The metabolic differences may be subtle and not sufficient to lead to recognizable alterations in the host cell, as in the case of mumps (76) and influenza B (77) virus in allantoic cells. In most instances, however, definite changes are produced in infected animal cells. These range from very minor pathological alterations to major changes in the infected cell and almost certainly are a reflection of the unusual biochemical events which arise from virus multiplication (78, 79, 80). In some instances the metabolic abnormalities associated with multiplication may be extensive enough to lead to destruction of the cell. This is especially evident with those animal viruses which cause cytopathogenic effects on multiplication in tissue culture systems (79, 81).

The appearance of recognizable abnormalities in the virus-infected cell is closely correlated with the emergence or release of new virus particles. This is evident from kinetic studies on virus multiplication in various tissue culture systems (71, 79, 81, 82, 83) and has been most elegantly demonstrated with single cells infected with poliomyelitis virus (52).

Whether it is the process of multiplication per se and the biosynthetic alterations involved or the products of the process that lead to damage of the infected cell is not yet clear. However, some viruses, when given in sufficiently large amounts, cause marked damage to cells even in tissues in which they do not multiply, as in the case of Newcastle virus in the mouse lung (84). With such agents, it appears that the virus particle itself possesses toxic properties (85). It is not yet known which component of the particle is responsible for toxicity. In addition, there is some evidence suggesting a direct correlation between the number of new particles produced during multiplication and the extent of cell damage. Viruses like influenza and Newcastle, which give a high yield of particles per cell (27), may cause damage sufficient to kill experimental hosts, while mumps, which gives a low yield (55), typically causes a relatively mild infection.

Tissue culture studies (79, 81, 82) suggest that recovery from cell damage associated with virus multiplication does not occur. However, investigations in the intact animal, especially with monkeys infected with poliomyelitis (86), indicate that obviously damaged cells may sometimes

recover. There is then a possibility that chemical modification of the reactions of the infected cell to multiplication or its products might be feasible. Almost nothing has been done in this interesting area. Were more known of the basis for the abnormalities produced in the infected cell, especially the changes which sometimes lead to its recovery, approaches to this problem would have more chance of success.

Reactions of Virus-Infected Tissues

Relation of virus concentration to tissue lesions.—In contrast to what occurs in the virus-infected cell, recognizable lesions do not appear in infected tissues for a considerable time after infection is initiated, usually not until the concentration of new virus particles has reached high levels. This relation is especially well observed in the mouse lung, infected with either influenza (34) or pneumonia virus of mice (70). The time curve of increase in virus concentration and that of increase in lung lesions are widely separated; the latter usually does not leave the base line until the virus curve has reached maximal levels. Even after peak concentrations of virus are present in the lungs, pneumonia may not appear until one to three days later (27). Similarly, in the spinal cord of the monkey, maximal concentrations of poliomyelitis virus are reached considerably before lesions or paralysis are seen (86).

Chemotherapy and tissue lesions.—By the time tissue lesions have definitely appeared, multiplication has usually progressed so far that presently available inhibitory compounds are incapable of altering the process (50). At this late stage, they usually do not diminish virus concentration and do not prevent further progression of lesions (28). Such compounds could hardly be designated as chemotherapeutic agents for they fail to modify the disease after it becomes recognizable from physical signs. The reasons for this failure are far from clear. Because of the short infective half-life of some viruses (27), it is necessary to assume that new particles continue to appear if the relatively constant concentration of infective particles in the plateau period is to be satisfactorily explained.

Direct tests have shown that when influenza virus is withdrawn from extracellular fluids it is promptly replaced from the infected tissue (87, 88). Presumably, therefore, multiplication may continue for long periods in some virus-host cell systems (89, 90). Why do compounds that effectively inhibit multiplication soon after the process has been started fail to cause inhibition, of what may be a similar process, at a later period? There is, as yet, no adequate answer to this question.

It is obvious that animal tissues represent a closely knit society of cells and are made up of a wide variety of cell types. It is probable that only one or a few types of cells in a given tissue support virus multiplication. A clear distinction between the infected and the noninfected cells in a tissue lesion is only rarely possible but it is probable that both infected and noninfected cells contribute to the eventual development of the pathological process.

In some instances, as in the mouse lung infected with influenza or other viruses, secondary reactions of inflammatory character dominate the pathological picture (91). In most instances, vascular reactions and cellular infiltrations occur in some degree (80). Even though they may not be prominent and almost certainly are secondary to reactions of the infected cells, they may make large contributions to the fully developed tissue lesion (80).

Chemotherapeutic investigations might equally well be directed toward modification of the reactions of tissues to virus infection as to inhibition of virus multiplication. From the viewpoint of the infected host, multiplication itself may be of little consequence if it does not lead to lesions or disease. A few studies appear to have moved in this direction, and some substances of biological origin appear to modify some secondary virus induced lesions without affecting virus multiplication (92, 93).

Recovery From Virus Disease

During the acute phase of virus disease, when lesions in infected tissues are present, the concentration of virus particles remains relatively constant, often at a high level. Eventually full recovery, recovery with persistent sequelae, or death occurs. In most virus diseases of man, recovery is the rule even though unfortunate sequelae persist after some (6). When recovery does occur both the virus concentration and the lesions progressively decrease. Eventually the virus as well as the lesions may entirely disappear. Kinetic studies have shown that decline in virus concentration precedes resolution of the lesions, which in some instances may persist for considerable periods (34, 70).

Mechanism of recovery.—The mechanism of the recovery process is but poorly understood. Relatively little study has been directed toward the phenomenon in recent years. In part, this reflects the common practice of using severe, usually fatal infections as laboratory models of virus diseases. Useful though such models are for many types of studies, they do not yield a faithful reproduction of many virus diseases as seen in nature. Although technically more difficult to handle, milder infections which are usually followed by recovery might prove to be revealing laboratory models. By careful choice of virus strains and routes of inoculation it is possible to induce in various small mammalian hosts, e.g., mice, hamsters, cotton rats, guinea pigs, and rabbits, nonfatal infections with a number of viruses, e.g., influenza, pneumonia virus of mice, lymphocytic choriomeningitis, herpes simplex, certain encephalitis agents, vaccinia, and dengue.

The natural recovery process is, in fact, a challenging phenomenon and close study of it might provide guides to effective chemotherapy. Artificial acceleration of the recovery process and the prevention of persisting damage to the host is the objective of treatment. Whatever the final explanation of recovery may be, it is probable that in many virus diseases the development of circulating antibody plays little if any part. As is well known, efforts to foster recovery by means of immune serum containing antibodies against

the virus have been unsuccessful (6). The fact that there is, in many instances, some correlation between the appearance of circulating antibodies and signs of recovery is not adequate evidence of a causal relation. Patients with agammaglobulinemia, who are unable to produce antibodies against the infecting agent, appear to recover from some virus diseases as readily and as promptly as normal persons (94, 95). Important as specific antibodies are in conferring immunity against certain virus diseases as well as in preventing migration through the blood to uninfected tissues, there is no adequate evidence that they can alter the course of an established virus disease.

Whether the virus becomes unable to continue multiplication because infected cells cannot indefinitely support the process, or for some other reason, the fact is that in the mammalian host, multiplication almost, if not entirely, ceases. It seems probable that this can be attributed to altered biosynthetic processes in the virus-infected cell which may not be beyond clarification. More information about this common and important phenomenon might prove highly rewarding.

PRINCIPLES OF INHIBITION OF ANIMAL VIRUS MULTIPLICATION

Basis of Specificity of Viruses

An understanding of the chemical basis for the biological specificity of nucleic acids might provide indications as to ways of interfering with the synthesis of virus nucleic acids without affecting host-cell nucleic acid synthesis. Unfortunately, such an understanding is lacking. No constituents have yet been found in animal virus nucleic acids which do not also occur in host cells (96, 97, 98). It may be inferred that the small molecular building blocks used in the synthesis of nucleic acids are common to host cells and animal viruses. Host cells appear to be capable of synthesizing these materials, since they can multiply in the absence of added purines, pyrimidines, ribose, and deoxyribose (99, 100, 101). Most of the energy required is provided by the mechanisms of oxidative and glycolytic phosphorylation involving a very large number of enzyme reactions (102, 103). Information is rapidly accumulating in regard to pathways of nucleotide synthesis (104 to 109) and enzymes have been found which catalyze the synthesis of high molecular polyribonucleotides from nucleoside diphosphates (110, 111) and polydeoxyribonucleotides from nucleoside triphosphates (112, 113). Nothing is as yet known about the sequence of nucleotides in natural nucleic acids (114, 115) or polynucleotides (111, 113) synthesized enzymatically in the test tube. A method suitable for determining the nucleotide sequence in ribonucleic acids has been proposed (116, 117), and its validity has been tested in a number of oligonucleotides (118). However, it cannot be applied to nucleic acids themselves until a truly homogeneous ribonucleic acid can be made available (115). Yet it seems likely that specificity of nucleic acids may reside largely in the nucleotide sequence, and that when nucleic acids are synthesized in cells this sequence is determined by the sequence in pre-

existing nucleic acid molecules. Reduplication of virus nucleic acids seems to be dependent on host cells not only for building blocks but also for energy (119, 120); many enzyme systems of the host are involved. Whether the animal host responds to virus infection with formation of certain enzymes that it does not produce normally is not known at present.

Somewhat similar considerations may be presented with respect to protein synthesis in the virus-infected cell. However, host cells are incapable of synthesizing all of the amino acids which go into proteins of various kinds and are thus dependent on an external supply of certain amino acids (121). Thus far, no amino acids have been found in animal viruses which do not exist in host cells (96). Recent evidence indicates that amino acids are activated prior to incorporation into protein molecules (122 to 125). It is of great interest that activation involves the formation of enzyme-bound amino acid$\sim$adenosine monophosphate compounds (124, 125). Nucleic acids may be of importance in determining the sequence of amino acids in proteins. That they play a role in protein synthesis is now well established (104).

Cell-Virus Relationship and Inhibition of Biosynthesis of Nucleic Acids and Proteins

In the absence of knowledge of specific chemical features of virus nucleic acids and proteins, approaches to inhibition of virus nucleic acid or protein synthesis are likely to be developed on the level of reactions which are probably common to host and virus (126, 127). The question arises immediately: is it possible that inhibition of nucleic acid or protein synthesis in a virus-infected cell, through interference with a step common to virus and host-cell nucleic acid or protein synthesis, may result in a marked degree of inhibition of virus multiplication in the absence of serious or irreversible damage to the infected cell or to as yet uninfected cells exposed to the same inhibitory agent? A definitive answer cannot be given at the present time. It is possible that the biosynthetic requirements of the multiplying virus are quantitatively different from those of the host cell (5). Furthermore, sites of synthesis of virus and host-cell materials may be differentially accessible to inhibitory compounds.

The biology and chemistry of virus-infected cells have received little attention and are very imperfectly understood. In the past, inhibition experiments have been restricted mostly to studies of effects on metabolic activities of cells or to uncorrelated studies of effects on virus multiplication. There is little information about alterations in the normal functions of uninfected or infected cells in the presence of compounds at virus inhibitory concentrations. The question of whether virus inhibition is due to a direct effect on synthesis of virus materials or, as is more likely in most situations, to a secondary effect resulting from inhibition of host-cell metabolism has been avoided by most investigators. It is probable that with few, if any, exceptions all compounds which have been used to inhibit virus multiplication have had effects on host-cell metabolism at virus inhibitory concentrations.

Since there is no evidence that viruses possess energy yielding mechanisms of their own, it follows that compounds which interfere with energy yielding mechanisms reduce the yield of virus wholly through effects on host-cell metabolism. Compounds which interfere with nucleic acid or protein synthesis may be assumed to reduce yield of virus through inhibition of virus nucleic acid or protein synthesis, although it is possible that concomitant reduction in synthesis of host nucleic acids or proteins may cause a secondary depression of the synthesis of virus materials. It seems likely that compounds thus far used to inhibit nucleic acid or protein synthesis in virus-infected cells have acted on the level of low molecular intermediates or precursors for both virus and host materials of high molecular weight. If the reactions on this level are catalyzed by host-cell enzymes, it is possible that present inhibitors of biosynthesis reduce the yield of virus through inhibition of host-cell processes. In the animal virus field, lack of evidence precludes the possibility of distinguishing between inhibition due to binding of sites on enzyme molecules and that due to incorporation of inhibitors into the end product (1).

These considerations point up the importance of determinations of effects of compounds on host-cell metabolic activities at virus inhibitory concentrations. Yet the opportunities which have presented themselves in virus multiplication experiments to obtain biochemical information on the mechanism of action of inhibitory compounds have rarely been utilized. Such information would bear not only on the biochemical requirements of virus multiplication but also on fundamental aspects of cellular metabolism.

Selective Inhibition

Many metabolic inhibitors are chemically highly selective in that they inhibit only certain reactions when the concentration used is appropriate. The degree of biological selectivity shown by such inhibitors depends on the importance of the inhibited reaction for the maintenance, functioning, and reproduction of the cell. If the reaction affected is of vital importance in cellular metabolism, and if no alternative pathways are open, high chemical selectivity may be associated with low biological selectivity. However, reversible inhibition of even a vital process may be followed by recovery of the cell from the induced metabolic derangement.

It is possible that at a given concentration of an inhibitory compound, inhibition of production of virus material might be greater than that of production of host material or that a certain degree of inhibition might have a more profound effect on the sustenance of the virus than the cell. Indeed, quantitative differences may be of paramount importance and could form the basis for selective inhibition of virus multiplication (126, 127). It is a truism that, given in sufficient amounts, almost all compounds including some natural amino acids are toxic. When the amount administered is increased, it is to be expected that in addition to a maximal primary effect and its consequences, other effects may take place. The question is: how high a

concentration is necessary for achieving the desired effect of virus inhibition in the absence of serious or irreversible damage to cells? It is well to remember that the difference between therapeutic and toxic doses with isoniazid, streptomycin, and many other drugs used effectively in the treatment of microbial and other diseases is approximately twofold.

In studies of the effects of inhibitory compounds on the metabolic activities of virus-infected cells it is not sufficient to consider effects in terms of chemical selectivity alone, although such thinking is essential if the mechanism of the primary action of inhibitors is to be understood. It is important to consider the infected cell as a whole. An adequate evaluation would require a large number of examinations of various kinds to establish the structural and functional soundness of the cell, or to discover any and all departures from normal.

Review of the literature shows that in many studies on inhibition of virus multiplication the cells were not examined, and in others examination was limited to visual inspection or determination of oxygen uptake. In some instances, more extensive study was made of effects of inhibitory compounds on cells (5, 54, 56, 57, 75, 77, 119, 120, 128 to 132).

The statement has been frequently made that various compounds were "active" as inhibitors of viruses in tissue culture but "ineffective" in experimental animals. It is suggested that if such compounds had been critically examined in tissue culture, with but few exceptions they would have been found to be toxic to cells at virus-inhibitory concentrations.

A better understanding and evaluation of the effects of inhibitory compounds on cells may be achieved by supplementing studies on inhibition of virus multiplication and on toxicity for uninfected cells with observations on reversibility of toxic manifestations upon withdrawal of the compound, and on protection of cells by the compound against virus cytopathogenicity.

One may ask: why not always use animals, and thus avoid the possibility of numerous pitfalls which exist in work with cells or tissues *in vitro*? Only a few of many considerations will be mentioned. If various substances or compounds are tested with the sole purpose of finding out whether they possess chemotherapeutic or chemoprophylactic usefulness, the experimental animal may be superior to *in vitro* systems. If, however, it is intended to relate the chemical structures of compounds to their biological and biochemical activities, to develop new and more potent compounds, and to undertake studies on the biochemical mechanism of action *in vitro* methods may prove more rewarding. If in such *in vitro* studies indications of a high degree of biological selectivity are found with certain compounds, they may then be profitably investigated in experimental animals.

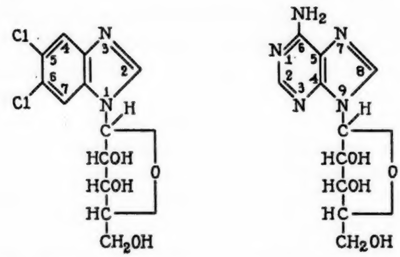
RECENT STUDIES WITH BENZIMIDAZOLE DERIVATIVES

Inhibition of Nucleic Acid and Protein Synthesis

Studies with benzimidazole glycosides (5) illustrate many aspects of the problem of inhibition of virus multiplication as seen from the viewpoint of

inhibition of biosynthetic processes. The structure of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, a new, highly active inhibitor of virus multiplication (75), and the time of its action early in the latent period of the multiplication cycle (51) suggested that this compound acts through inhibition of RNA synthesis (5, 51, 75, 133). This prediction has been borne out in experiments on incorporation of adenosine-8-C^{14} into RNA of the host tissue (56, 57) and of orotic acid-6-C^{14} into RNA of isolated calf thymus nuclei (58).

Figure 1 depicts the structural relationship between 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, the inhibitor, and 1-β-D-ribofuranosyladenine (adenosine), present in RNA, in adenosine mono-, di-, and triphosphate, and in several coenzymes. The pentose moiety is identical in the two compounds.



5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (Inhibitor) | 9-β-D-Ribofuranosyladenine (In RNA)

FIG. 1. Comparison of the structure of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and 9-β-D-ribofuranosyladenine (adenosine).

Both possess a bicyclic skeleton of the same size, and in each, one of the ring structures is 5-membered imidazole. The 6-membered benzene ring structure of the benzimidazole ribofuranoside, with its chlorine substituents, is markedly different from the pyrimidine ring structure in adenosine. Although it was reported (75) that a mixture of adenosine, adenylic acid, guanosine, and guanylic acid failed to block the inhibitory effect of the 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole on influenza virus multiplication, it was shown that adenosine alone is capable of blocking the inhibitory effect of the compound (56). It should be emphasized that this occurred only when a low concentration of the ribofuranoside of dichlorobenzimidazole was used to give 50 to 75 per cent inhibition of virus multiplication. At higher concentrations of the compound, adenosine was incapable of blocking inhibitory action. Guanosine was ineffective.

Chorioallantoic membrane.—At virus-inhibitory concentrations, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole caused reduction in the uptake of

adenosine-8-C^{14} into RNA of the uninfected chorioallantoic membrane from 10 to 11-day-old embryonated eggs (56). The degree of inhibition of uptake of the labeled precursor was directly related to concentration of the 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Inhibition of RNA synthesis was observed after a 3-hr. period of incubation of chorioallantoic membrane with ribofuranoside of dichlorobenzimidazole and adenosine-8-C^{14} *in vitro* under conditions of virus-inhibition experiments.

In single-cycle experiments with influenza virus, processes inhibitable by the 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole were limited to a 3-hr. interval from virus inoculation (51). Within this interval, the inhibitory effect of the substrate on virus multiplication decreased as the time between virus inoculation and introduction of compound was increased. For the production of soluble, complement-fixing antigen, processes inhibitable by ribofuranoside of dichlorobenzimidazole were of even shorter duration and no significant effect on the production of soluble antigen was seen when the inhibitor was given 1 hr. after inoculation. Since processes inhibitable by this compound ceased before soluble complement-fixing antigen or virus particles had been produced in demonstrable amounts in infected tissue, it was concluded that ribofuranoside of dichlorobenzimidazole interfered with the synthesis of precursor substances, nucleic acid in nature, for both these virus materials (51). The fact that it did not affect virus multiplication when given during the incremental period in single-cycle experiments suggests that inhibition of the host RNA synthesis during this period is of no consequence to the multiplying virus.

5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole showed only a slight effect in some experiments and no effect in others on incorporation of C^{14}-L-alanine into the protein fraction of the chorioallantoic membrane (56). This result does not necessarily indicate that RNA was of little or no importance for protein synthesis in the membrane; rather, it is likely that the RNA, which was playing a role in protein synthesis during the 3-hr. period of incubation, was already present in cells at the beginning of the experiment.

Monkey kidney cells.—Two glycosides of 5,6-dichlorobenzimidazole were used to delineate the duration and chemical nature of the biosynthetic processes involved in poliovirus type 2 multiplication in single-cycle experiments in monkey kidney cells (57) maintained in Eagle's medium without protein (121). In addition to the β-D-ribofuranoside, the α-D-arabinopyranoside of 5,6-dichlorobenzimidazole was employed. Of the two compounds the latter was less toxic than the former for monkey kidney cells at equivalent virus-inhibitory concentrations (134, 135). It should be emphasized that these compounds were used at equivalent inhibitory concentrations for poliovirus type 2, determined in multiple cycle experiments in which virus and compound were introduced simultaneously (134, 135). Each compound was used at a concentration which caused 95 per cent reduction in the yield of virus (135). Since the ribofuranoside was 11 times more active, on a molar basis, as an inhibitor of poliovirus type 2 multiplication (135), the concen-

tration of the arabinopyranoside of dichlorobenzimidazole employed (0.00107M) was 11 times greater than that of the ribofuranoside (0.000095M).

In single-cycle experiments in stationary tube cultures, the ribofuranoside or the arabinopyranoside of dichlorobenzimidazole was given simultaneously with virus or at various times after inoculation of virus (57). When compound and virus were given simultaneously the arabinopyranoside caused 95 per cent reduction in yield, whereas the ribofuranoside caused 60 per cent reduction. Thus, the arabinopyranoside was as effective in single-cycle experiments as in multiple-cycle experiments, whereas the ribofuranoside was somewhat less effective in single-cycle experiments. As the interval between inoculation of virus and introduction of compound was increased, the inhibitory effect decreased; that of the ribofuranoside decreased more rapidly than that of the arabinopyranoside. When the interval was 5 hr., yield of new virus was not reduced by the former, whereas the latter still caused a moderate reduction in yield.

The effect of these compounds at equivalent virus-inhibitory concentrations on RNA and protein synthesis was determined with uninfected cell suspensions which were incubated and shaken for 3 hr. in the presence of adenosine-8-C^{14} or C^{14}-L-alanine (57). Both the ribofuranoside and the arabinopyranoside inhibited the uptake of adenosine-8-C^{14} into RNA; the effect of the β-D-ribofuranoside was slightly greater (57 per cent inhibition) than that of the α-D-arabinopyranoside (45 per cent inhibition). On the other hand, uptake of C^{14}-L-alanine into protein was inhibited to a much greater extent by the arabinopyranoside (62 per cent) than by the ribofuranoside (28 per cent). Thus, the biochemical action of the latter as an inhibitor of RNA synthesis was correlated with the kinetic finding that the greater part of its effect on poliovirus multiplication took place early during the latent period. In contrast, the arabinopyranoside caused marked inhibition of protein synthesis and its virus inhibitory effect was manifest throughout the whole latent period and even beyond (57).

Isolated calf thymus nuclei.—Incorporation of orotic acid-6-C^{14} into the pyrimidines of RNA of isolated calf thymus nuclei was markedly inhibited by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (58). The synthesis of RNA could be inhibited at any time by this derivative. However, the ribofuranoside inhibited the synthesis of proteins only if present at the outset of incubation. If added 30 min. later it had no effect on incorporation of alanine-1-C^{14} into the proteins of isolated thymus nuclei. It appears that incorporation of amino acids into proteins requires a preliminary activation of the isolated nucleus, and that this is accomplished by synthesis of RNA. Once the activation had occurred the ribofuranoside failed to inhibit further amino acid uptake into proteins. Thus, continued synthesis of proteins may take place in the presence of the ribofuranoside at concentrations markedly inhibitory to RNA synthesis.

In view of the facts that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

caused no or only slight inhibition of uptake of C^{14}-L-alanine into proteins of the chorioallantoic membrane even when present at the outset of incubation (56), and that it did cause a moderate degree of inhibition of protein synthesis in monkey kidney cells (57) and isolated calf thymus nuclei (58), the question arises whether or not its effect on protein synthesis, when seen, was due to a partially depleted state of calf thymus nuclei and monkey kidney cells in contrast to the state of chorioallantoic membrane freshly removed from embryonated eggs. Preparation of cell nuclei and monkey kidney suspensions takes time and involves a number of steps including several washings. However, these considerations do not detract from the fact that in the ribofuranoside of dichlorobenzimidazole (75) a new inhibitor of RNA synthesis has been discovered (56, 57, 58).

Inhibition of Virus Multiplication

Historical background.—The first benzimidazole (136) was prepared in 1872 by Hobrecker (137) who synthesized the 2,5-dimethyl derivative. In 1944, Woolley (138) considered benzimidazole to be a structural analogue of purines and used it in studies on inhibition of multiplication of yeast and bacteria. Three years later, Thompson (139) reported that benzimidazole inhibited vaccinia virus multiplication in minced chick embryo tissue *in vitro*. No data were given in regard to effects on cells. In 1950, Rafelson and associates (128) used benzimidazole to inhibit Theiler's GDVII mouse encephalomyelitis virus in minced brain tissue, and showed that benzimidazole inhibited incorporation of radioactive phosphorus into phospholipids and the "protein-bound" fraction of the brain. In 1952, Morgan (140) reported that benzimidazole inhibited the multiplication of the psittacosis agent in minced chick embryo tissue. In the same year, Brown (141) found that benzimidazole was inhibitory to poliovirus multiplication in monkey testicular tissue *in vitro*. Possible effects of benzimidazole on host cells were not reported. In all of these studies the inhibitory activity of benzimidazole was low; about 0.25 to 1.0 mg./ml. was required to cause significant reduction in yield of virus. Also in 1952, 2,5-dimethylbenzimidazole was shown by Tamm *et al.* (142) to inhibit influenza B virus in the chorioallantoic membrane *in vitro* without affecting oxygen uptake of the membrane (142). The 2,5-dimethyl derivative was 2.7 times more active than unsubstituted benzimidazole (74).

Development of new derivatives.—The finding that 2,5-dimethylbenzimidazole inhibits influenza B virus multiplication (142) was the first step in a series of investigations which have led to the synthesis[4] of new highly active and moderately selective glycosides of benzimidazoles (5, 75, 77, 126, 133, 143). In the development of new derivatives the importance of quantitative biological studies in guiding chemical synthesis has been apparent (5, 126).

[4] The benzimidazole derivatives were synthesized by Dr. Karl Folkers and Dr. Clifford H. Shunk of Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

Quantitative *in vitro* procedures were developed for the determination of both virus inhibitory activity (73) and toxicity of compounds (75, 77). These procedures made it possible to correlate chemical structure with biological activity with very small amounts of compounds unhampered by problems of absorption, transport, and excretion (5).

Examination of compounds.—Effects of benzimidazole derivatives on virus multiplication (73, 74, 75, 132 to 135, 141 to 145), and on biochemical processes (56, 75, 77, 132) and morphological characteristics (77, 134, 135) of host cells are markedly dependent on the concentration of the compound used. Quantitative measurements of the activities of various derivatives have been based on determinations of the molar concentration required to cause a certain defined effect (5). A series of concentrations of each compound was employed and the effect at each concentration was determined; then curves were constructed relating concentration of compound to magnitude of effect and interpolation made of the molar concentration at which a certain defined effect, such as 75 per cent reduction in yield of virus, would occur. The interpolation was made within a straight line portion of the curve.

Such a procedure permits precise measurement of the various effects of interest. In a large number of determinations of inhibitory activities of benzimidazole derivatives on influenza B virus multiplication, the mean of standard deviations obtained with different compounds was 12.0 per cent (74, 75, 133). Toxicity of benzimidazole derivatives for the chorioallantoic membrane *in vitro* was estimated on the basis of characteristic macroscopic changes in the appearance of the membrane (77). These correlated well with reduced oxygen uptake by the membrane (77). With monkey kidney cells, toxicity was determined on the basis of microscopic examination (134, 135).

The ratio of the cytotoxic to the virus-inhibitory concentration provides an indication of biological selectivity of action (5, 77, 126, 134, 135). The validity of such ratios is presently limited to comparison of different derivatives with different viruses in the same host-cell system.

With 2,5-dimethylbenzimidazole and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, the difference in the degree of selective action found on the basis of inspection of chorioallantoic membranes was confirmed in studies on; (*a*) oxygen uptake by membranes (75, 77); (*b*) microscopic appearance of membranes (77); and (*c*) inhibition of proliferation of cells of the membrane in roller tube cultures (75). In all these experiments 2,5-dimethylbenzimidazole was found to be less selective than the ribofuranoside of dichlorobenzimidazole. Finally, the latter inhibited influenza B virus multiplication in embryonated eggs and mice without serious effects on the host (75, 143), whereas the 2,5-dimethyl derivative failed to do so (126).

Structure-activity relationships in virus inhibition.—Unsubstituted benzimidazole was used as the reference compound in all studies with benzimidazole derivatives to be described (75, 77, 133, 134, 135, 143, 144, 145). Figure 2 summarizes some important relationships between chemical structure and influenza B virus inhibitory activity (75, 133, 143). The inhibitory

activity of benzimidazole derivatives increased with multiple substitution of chlorine atoms in the benzene ring. Furthermore, the β-linked D-ribofuranosides of these chlorobenzimidazoles were much more active than the corresponding chloro derivatives. When the glycosidic ring was changed from the 5-membered D-ribofuranoside in β-linkage to the 6-membered D-ribopyranoside in α-linkage[5] inhibitory activity dropped to $\frac{1}{6}$ of that found for the ribofuranoside. In fact, the activity of the ribopyranoside was almost identical to the activity of the simple 5,6-dichloro compound. The activity of the

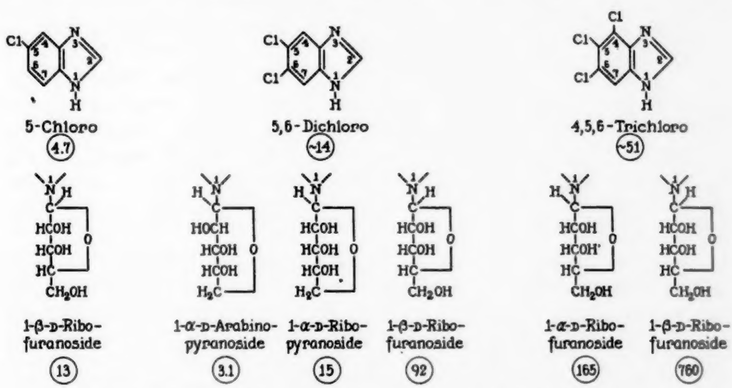


FIG. 2. Chemical structure and influenza B virus inhibitory activity of benzimidazole derivatives. Unsubstituted benzimidazole causes 75 per cent inhibition at 0.0035 *M;* activity = 1. The encircled numbers below each compound refer to relative activity. Data from Tamm (143) and Tamm *et al.* (75, 133).

α-D-arabinopyranoside[5] was even lower indicating that substitution of this pentose depressed the activity of the chloro compound. Comparison of the α and β forms of the ribofuranoside of trichlorobenzimidazole showed that the β-linked ribofuranoside was much more active than the α form.

It has been reported that nucleic acid in influenza virus is of the ribose type (96, 98, 146, 147). In nucleic acids, ribose occurs in β-linked furanose form (114). Thus, the highest virus inhibitory activity is obtained when the carbohydrate moiety is identical with that present in the nucleic acid of influenza virus particles and when the benzene ring contains numerous halogen substituents.

With vaccinia virus however, which contains DNA rather than RNA (96, 148, 149), substitution of β-D-ribofuranose in the imidazole ring did not increase the inhibitory activity of chlorobenzimidazoles (145). This finding

[5] In certain reports (5, 58, 75, 77, 134, 143), 5,6-dichloro-1-α-D-arabinopyranosylbenzimidazole and 5,6-dichloro-1-α-D-ribopyranosylbenzimidazole were erroneously identified as the β-linked isomers.

agrees with the observation that the ribofuranoside of dichlorobenzimidazole did not appreciably inhibit DNA synthesis in isolated calf thymus nuclei in concentrations at which RNA synthesis was markedly reduced (150).

On the other hand, the structure-activity relationships determined for influenza and poliomyelitis viruses, both of which contain RNA (98, 151), with unsubstituted benzimidazole and 5 glycosides of chlorobenzimidazoles were closely similar (134, 135). The most active derivatives against both viruses were β-linked ribofuranosides of chlorobenzimidazole. Any departure from the β-D-ribofuranose structure in the carbohydrate moiety of the benzimidazole glycoside resulted in reduced inhibitory activity against both influenza and poliovirus multiplication.

The observed structure-activity relationships suggested (133) that halogenated β-D-ribofuranosylbenzimidazoles interfere with RNA synthesis directly rather than through mediation of vitamin B_{12}. The similarities and differences shown by influenza, vaccinia, and polioviruses in susceptibility to inhibition by benzimidazoles probably reflect the nature of nucleic acid in the virus particle, i.e., RNA in influenza and poliomyelitis viruses; DNA in vaccinia virus.

Structure-activity relationships in regard to selective action.—An apparent difference was found in regard to the selective action of benzimidazoles as inhibitors of influenza and poliomyelitis virus multiplication. With influenza virus β-linked D-ribofuranosides of halogenated benzimidazoles were not only the most active but also the most selective inhibitors (77, 126). However, with poliovirus the β-linked ribofuranosides were no more selective than unsubstituted benzimidazole although they were highly active as inhibitors (134, 135). The α-linked D-arabinopyranoside of 5,6-dichlorobenzimidazole was the most selective compound with poliovirus, i.e., it was 1.6 times more selective than unsubstituted benzimidazole. With influenza virus the α-linked arabinopyranoside was no more selective than unsubstituted benzimidazole.

It is not possible to offer an explanation for these findings at the present time but apparent differences in selective action might largely disappear if effects of benzimidazoles on the multiplication of influenza and poliomyelitis viruses were studied under more comparable conditions. Three differences between the host-virus systems used appear of greatest importance: (*a*) Influenza virus does not cause cytopathogenic changes in the cells of the chorioallantoic membrane (77), whereas poliovirus is highly cytopathogenic for monkey kidney cells *in vitro* (81). (*b*) In the chorioallantoic membrane toxicity of compounds was determined on the basis of macroscopic damage (77) whereas in the studies with monkey kidney cells toxicity of compounds was determined by microscopic examination (134, 135). (*c*) The biosynthetic processes in the chorioallantoic membrane and monkey kidney cells may be different in certain respects since 5,6-dichloro-1-β-D-ribofuranozylbenzimidazole caused a moderate degree of inhibition of uptake of C^{14}-L-alanine into

monkey kidney cell proteins (57) but failed to show such an effect in the chorioallantoic membrane (56).

Virus-inhibitory activity and tissue toxicity of different derivatives may vary independently (5, 77, 126, 134, 135). Of greatest interest are the findings that alteration of the structure of an inhibitor molecule may increase the virus inhibitory activity more than toxicity, or it may reduce the toxicity without affecting virus-inhibitory activity. In both cases compounds of increased selectivity are obtained. This is true both for the glycosides of benzimidazoles as well as derivatives of other kinds.

The most active compound so far developed is 5-(or 6-)bromo-4,6-(or 5,7-)dichloro-1-β-D-ribofuranosylbenzimidazole (133). It causes 75 per cent inhibition of influenza B virus in the chorioallantoic membrane *in vitro* at a concentration of 1.8×10^{-6} M or 0.72 μg./ml. Its potency is comparable to that of sulfonamides and other antimicrobial substances on bacterial multiplication. Experiments with influenza B virus in the mouse lung have shown that β-D-ribofuranosides of halogenated benzimidazoles are capable of inhibiting Lee virus multiplication in the mouse lung (75, 133) and of prolonging the survival time of infected mice (152) when administration of compound is started 2 hr. after inoculation of virus. However, these compounds are not sufficiently selective to be used in sufficient amounts to cause marked inhibition for a period of one week or longer without damage to the host (133).

Unsubstituted benzimidazole caused a slight reduction in the mortality of mice inoculated with a small dose of the Lansing strain of poliovirus type 2 (153). Yet at concentrations inhibitory to the multiplication of the Mahoney strain of poliovirus type 1, unsubstituted benzimidazole caused a reduction in the oxygen uptake of HeLa cells (132). It is possible that a greater reduction in mortality of mice infected with Lansing virus might be obtained with 5,6-dichloro-1-α-D-arabinopyranosylbenzimidazole which is not only four times more active, but also somewhat more selective, than unsubstituted benzimidazole in monkey kidney cells *in vitro*. However, it should be emphasized that the effect originally obtained with unsubstituted benzimidazole was very slight and that the difference in selectivity between the new derivative and unsubstituted benzimidazole is small (134, 135).

There is a need for more selective inhibitors of virus multiplication. It is apparent that selective inhibition of virus multiplication requires compounds of a special kind (5, 144). Many of the benzimidazole derivatives, and many other compounds which have been examined lack the properties which would recommend them for more extensive virus studies (5, 144). Only a beginning has been made in the study of selective inhibition of virus multiplication. As to benzimidazole derivatives, the possibilities for improvement through investigation of new derivatives have by no means been exhausted.

RECENT STUDIES NOT PREVIOUSLY REVIEWED

In experiments with poliovirus type 1 in cynomolgus monkeys, approximately 100 ID_{50} of virus was inoculated subcutaneously (154). Helenine, an

acetone precipitate derived from culture filtrates of *Penicillium funiculosum*, was injected intraperitoneally; two doses were given the day before virus, the third dose 4 hr. before and the fourth dose 4 hr. after virus inoculation. The incidence of paralysis was reduced from 100 per cent in the control group to 18 per cent in the helenine-treated group. The average time to the onset of paralysis in those helenine-treated monkeys that eventually developed paralysis was 23.5 days as against 8.8 days in control monkeys.

In view of the apparent effectiveness of helenine as a prophylactic agent, an attempt was made to detect therapeutic activity. Ten monkeys were infected subcutaneously and rectal temperatures were recorded daily. When an animal exhibited a temperature of 104° F., or showed evidence of paralysis, treatment was initiated. Alternate animals in order of onset received helenine twice daily for not more than four days, or equal volumes of buffered saline intraperitoneally. The group which was treated with helenine and the group of control animals each had an average survival time of 2.6 days after the detectable onset of disease. Thus, helenine showed no therapeutic activity. In experiments on prophylaxis with poliovirus type 2 in mice, helenine did not alter significantly the incidence of paralysis, although it did appear to prolong the incubation period.

Synthetic polylysine peptides (155) possess inhibitory effects for influenza A (156), mumps (157, 158), Newcastle, and infectious bronchitis viruses (159). These basic, high-molecular weight lysine peptides are also capable of inactivating tobacco mosaic virus by direct combination with the virus (160), of inactivating a bacteriophage (161), and of agglutinating chicken red blood cells (162). Inactivation of tobacco mosaic virus is partially reversible on dilution of the reaction mixture (160). The ability of polylysine to inactivate bacteriophage is neutralized by DNA and RNA (161). It is probable that this is due to combination between polylysine and DNA or RNA. Allantoic fluid inactivates the hemagglutinating activity of the polypeptide. This inactivation is probably emzymatic (158, 162).

In experiments with influenza A virus in the allantoic sac of embryonated eggs (156) the size of the inoculum was 10^5 EID_{50} and various synthetic polypeptides were injected either 1 hr. before, together with, or 3.5 hr. after virus. In some cases polypeptides were injected initially and again 24 hr. later. Concentration of virus in the allantoic fluid was measured at 24 or 48 hr. At 24 hr., inhibition was evident when the polypeptides were injected before or with the virus. When the polypeptides were injected after the virus, less inhibition was obtained. At 48 hr. no significant inhibition was seen even against smaller virus inocula of 10^3 EID_{50}. Repeated doses of the polypeptides initially and 24 hr. after inoculation, also failed to reduce the amount of virus produced by 48 hr. The authors interpreted their findings as indicating that basic polypeptides combined directly with virus particles to inhibit infectivity. It is likely that such was the case. Preliminary experiments failed to show a significant chemotherapeutic effect against influenza virus in mice when the polypeptides were administered as an aerosol.

Polylysine, injected into the allantoic sac 1 hr. prior to inoculation of 10^2–10^3 LD_{50} of a strain of infectious bronchitis virus in early egg passage, protected 80 per cent of the infected embryos against death (159). The experiment was terminated on the sixth day at which time all of the control embryos were dead. Injection of polylysine 1 hr. after inoculation of 10 LD_{50} of virus also showed a marked protective effect. After the virus had been passed in eggs a number of times less protection was demonstrated. The degree of protection depended markedly on the strain used and with one strain no protection was demonstrated in eggs infected with only 10 LD_{50} of virus. Polylysine protected against 6 LD_{50} but not 100 LD_{50} of Newcastle virus when the compound was given 1 hr. before the virus. The authors again suggested that polylysine inhibits by combination with acidic groups on the virus surface. With the very small inocula used, polylysine was probably able to prevent the few infective virus particles introduced into the relatively large allantoic cavity from reaching the chorioallantoic membrane while still infective.

In studies with mumps virus (157) polylysine, of molecular weight 2400, injected 1 hr. before the virus, caused a significant reduction in yield as measured six days later even when as much as 3×10^5 EID_{50} of virus was inoculated. When only 30 EID_{50} of mumps virus was inoculated 1 hr. after the compound, polylysine of molecular weight 2400 was effective at the 10 μg. level but not at the 1 μg. level. Polylysine, of molecular weight 16,000, was even more effective in that reduction in yield was obtained with 1 μg. Employing 200 μg. of polylysine of the smaller molecular size, inhibition became less striking as the interval between virus inoculation and injection of compound was increased beyond 36 hr., and no inhibition was demonstrated when the polypeptide was injected 56 hr. after virus. Injection of 400 μg. of polylysine, of molecular weight 2400, into the yolk sac 1 hr. before inoculation of 30 EID_{50} of virus into the allantoic cavity caused marked inhibition. Polylysine of 16,000 molecular weight was much less inhibitory under these conditions. When the smaller polypeptide was inoculated into the yolk sac 26 hr. prior to allantoic injection of virus no significant inhibition was obtained. In an experiment on direct inactivation of mumps virus, mixtures of polylysine and virus were held at 3° C. for 30 min. Under these conditions polylysine did not cause inactivation of infectivity. The mechanism of action of polylysine was discussed at considerable length. The explanation which seemed most likely to the authors concerned the possibility that polylysine might act intracellularly by diffusing into the cell and combining with intact virus, virus fragments, or intermediates in virus formation within the cell. It would appear however, that the evidence offered does not exclude the possibility that the effects observed were due to extracellular combination between polylysine and mumps virus which rendered the virus noninfective.

All of these results led Stahmann and associates (163) to test the possibility that in the basic polyelectrolyte the polypeptide backbone may not be essential for inhibitory activity. Indeed, polyvinylamine, a synthetic basic

polyelectrolyte of unknown molecular size, was active against influenza B, mumps, and Newcastle viruses but its activity was less than that of polylysine examined under the same conditions.

It has also been shown that poly-D-lysine, although ten times more toxic, inhibits mumps and influenza B viruses to approximately the same extent as poly-L-lysine (158).

In experiments with amethopterin or guanozolo, and lymphocytic choriomeningitis virus (164), 10 LD_{50} of virus was inoculated intracerebrally in mice and the compound was given intraperitoneally, starting one day after virus inoculation. The period of observation was 14 days at which time all control mice were dead. Amethopterin was more effective than guanozolo in protecting a proportion of infected mice against death. Both compounds were more effective in C×DBA than Swiss mice. All of the treated mice showed evidence of toxicity from drugs before illness from infection had time to develop. When the frequency of injections of amethopterin was reduced from daily administrations to injections every other day, better survival of intracerebrally-infected Swiss mice was obtained. Infection was not eradicated by the drugs and surviving mice had prolonged periods of viremia.

Theiler's GDVII mouse encephalomyelitis virus was used in inhibition studies in minced brain suspensions (165). Approximately 100 intracerebral LD_{50} of a tissue culture strain of GDVII was inoculated per 50 to 100 mg. of tissue. The amount of virus present in supernatant after two days incubation was determined. Certain pyrimidine-related compounds inhibited GDVII under these conditions. Inhibition by 5-hydroxyuridine was partially reversed by uridine-5′-phosphate. Two of the four thiosemicarbazones tested were also inhibitory, as was glutamine. Chlorpromazine was as inhibitory as any drug tested against GDVII in minced mouse brain suspensions (165). Five μg. of this compound caused a 50 per cent reduction in yield of virus. Chlorpromazine did not protect mice from death resulting from intracerebral infection with 1 to 10 LD_{50} of GDVII virus. No experiments were reported on effects of the various compounds on host cells or on host-cell metabolic activities.

In a study with 44 compounds (166) an attempt was made to determine the virus-inhibitory effect on the basis of prevention of typical cytopathic changes in fibroblasts caused by types 1, 2, and 3 poliovirus. No reference was made to cytotoxicity of the compounds used. The amount of virus produced was not determined. The authors concluded that most compounds were equal in their effect against types 2 and 3 but some were less effective against an equal number of infective doses of type 1. It is possible that the development of virus lesions was prevented in many cases, not because of reduction in virus multiplication but because cytotoxic effects of the compounds themselves interfered with the development of virus lesions. Furthermore, the possibility that certain of the compounds used inactivated poliovirus was not excluded.

APPENDIX

Since the completion of this review, a paper appeared by Kissman *et al.* (167) which requires comment because of the nature of statements made by these authors. To correct erroneous impressions created it should be emphasized that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole has been and is available to interested investigators (58, 168).

On the basis of experiments on survival of mice infected with the PR8 strain of influenza A virus, Kissman *et al.* (167) claim that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole has no effect on the multiplication of influenza virus. In the same report (167), it is stated that this compound and the β- and α-linked isomers of 1-(5-deoxy-D-ribofuranosyl)-5,6-dichlorobenzimidazole showed no ability to prevent the multiplication of the Mahoney strain of poliomyelitis type 1 virus in HeLa cells *in vitro* at the maximum tolerated dose. However, this conclusion was based entirely on observations on the development of cytopathic changes. Thus, the authors have drawn conclusions in regard to virus multiplication without determining the amount of virus produced.

The authors also question the validity of the findings of Tamm *et al.* (75) that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole inhibits the multiplication of the Lee strain of influenza B virus in the chick embryo and in mice. They suggest that hemagglutination inhibition is not a valid measure of virus multiplication (167). It appears that they are confusing virus hemagglutination-inhibiting activity of compounds with inhibitory effect on virus multiplication. It should be emphasized that the ribofuranoside of dichlorobenzimidazole does not inhibit agglutination of chicken erythrocytes by Lee virus (75). In studies on inhibition of Lee virus multiplication by this compound, yield of virus was frequently measured by the hemagglutination technique and, in addition, the yield was measured by determining the amount of infective virus produced (75). Inhibition of virus multiplication by the ribofuranoside of dichlorobenzimidazole was demonstrable regardless of whether the virus yield was measured by hemagglutination or infectivity titrations (75). Various undocumented statements made by Kissman *et al.* (167) regarding other viruses are not discussed here.

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ANTIBODIES AGAINST ENZYMES[1,2]

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Immunology was largely developed from studies of the inhibition of toxins and hence from experiments which considered the interplay of three factors: toxin, antibody and the organism affected by the toxin. Survival or death of the organism is the indicator of this immune reaction. The complexity of such a highly organised test system, as exemplified by the importance of the permeability of tissue barriers (1, 2), makes it difficult to study the fundamental aspects of the reaction between toxin, antitoxin, and the tissue receptor on which the toxin acts. A major advance was therefore made when a simplified system, the reaction *in vitro* of antigen and antibody, was investigated. Through such studies specificity was understood in detail, precise analytical methods were introduced into immunology, and molecular ratios of antigen to antibody were determined for many antigens over a wide range of relative proportions.

Studies considering only two components, antigen and antibody are, however, unable to answer many of the questions posed by the immunology of toxins. It is, for instance, very difficult to neutralise toxin if it is injected simultaneously with antitoxin but at a different site. A dose of antitoxin which would protect completely if incubated with the toxin before injection is totally inadequate to protect against toxin injected separately. It is this type of problem [see also (105)] which could be examined *in vitro* by the study of immunological systems in which a third parameter is available, a situation which arises when the antigen is an enzyme.

This may serve as part of a justification for investigations of the interaction between antibody, enzyme, and substrate. To advance such studies an understanding must be gained of the mechanism by which antibodies inhibit enzyme action. More detailed work on selected systems will be needed to achieve a synthesis between the existing extensive information on antigen-antibody interaction and on enzyme-substrate interaction. Once this has been accomplished, it should be possible to add to the understanding of the interaction between enzyme and substrate. It is already possible to describe some examples in which a study of enzyme-antibodies has provided a useful tool. These and some potential applications will be mentioned. Aspects of recent papers will be reviewed to indicate experiments from which the mechanism of the inhibition of enzymes by antibodies may be gradually revealed. No attempt will be made to review the earlier literature completely, since this has been done by a book [Sevag (3)] and by two reviews which have appeared in recent years [Marrack (4); and Sevag (5)].

[1] The survey of literature was concluded in December, 1956.

[2] The following abbreviations have been used: ATP (adenosine triphosphate); DPN (diphosphopyridine nucleotide); DPNH (reduced diphosphopyridine nucleotide).

Enzyme-antibody Studies as a Tool in Biological and Immunological Investigations

Immunological methods are of great potential value for studies of the dynamic aspects of protein synthesis, of the fate of enzymes lost in the course of evolution [urease, allantoicase, uricase; (6)] and of such pathological variations as may be due to the loss or to the changed properties of enzyme systems. This potential has, so far, only been realised in studies on the synthesis of induced enzymes, particularly in the analysis by Cohn, Monod & Torriani of the synthesis by *Escherichia coli* of induced β-galactosidase (Gz).

Antibody to induced β-galactosidase was found to be removed by extracts from *E. coli* which were not adapted and possessed negligible amounts of β-galactosidase activity (7). The specific antibody was, therefore, removed by an antigen immunologically similar to Gz but without enzyme activity; this antigen was called Pz. Gz was precipitated preferentially by Gz antisera and Pz was precipitated only after all of Gz was removed. The quantity of Pz antigen could thus be determined in extracts containing both Pz and Gz by finding the equivalence points in mixtures containing a constant volume of Gz antisera and varying volumes of a mixture of Gz and Pz. Two different equivalence points could be observed, in terms of antigen and antibody in the supernatant as measured by (*a*) precipitin techniques and (*b*) enzyme assays of the supernatant. The difference between the concentrations of antigen corresponding to each of these two equivalence points represented the concentration of Pz in the antigenic mixture (8).

By the use of this method the effect of induction on the combining units of Gz and Pz per mg. N of cell extract could be followed in continuous culture. While Gz increased, the concentration of Pz fell. The following possibilities for the relation of the two proteins were considered (9): (*a*). Pz is a precursor of Gz; (*b*) Pz and Gz have a common precursor; (*c*). Pz and Gz are independently synthesized by similar mechanisms. The first of these possibilities became unlikely (10) when it was shown that pre-existing proteins of the cell contributed very little, if at all, to the composition of the induced enzyme. Induction resulted in the complete synthesis from amino acids of a new protein. It could, therefore, be concluded that Pz and Gz had an unstable common precursor or were independently synthesised by a similar mechanism.[3]

A detailed study of the immunology of penicillinases of *Bacillus cereus* led Pollock (11, 12) to the discovery of a cell-bound penicillinase of which only 30 to 50 per cent could be neutralised by antiserum to the exo-penicillinase. The fraction which could not be neutralised and could be extracted into solution after disintegration of the organism has been called γ-penicillinase. The enzyme is inducible. The proportion of penicillinase activity that cannot be neutralised forms a constant proportion of the total penicillinase activity. In view of this fact and because the Michaelis constant for γ is the same as

[3] Independent experiments on the induced synthesis of maltase by yeast have led to a similar finding (108).

for the other penicillinases, Pollock has proposed that these enzymes may have a common biochemical pathway in some part of their synthesis.

One of the most important contributions of quantitative immuno-chemistry has been the determination of the molecular ratio of antigen to antibody in precipitates formed by these reactants. Such determinations are based on the precipitation of all the antigen in the zone of antibody excess and become impossible if a substantial part of the antigen remains in solution. The presence of antigen and antibody in the same supernatant is taken as proof of antigenic inhomogeneity. Recent experiments with radioactively labelled antigens (13, 14), have led to the conclusion that soluble antigen-antibody complexes can exist throughout the zone of excess antibody as well as throughout the zone of excess antigen. Such complexes may be difficult to detect by conventional immunological procedures. However, it is conceivable that the persistence of radioactivity in supernatants is due to inhomogeneous labelling, so that one small fraction of antigen is so highly labelled that it reacts as a heterologous cross-reacting antigen.

An enzyme which is not completely inhibited by antibody would permit the detection of such soluble complexes without any chemical modification of the antigen (15). Investigations into the reaction of ribonuclease A and its antisera obtained at various stages during prolonged courses of immunisation, led to the discovery of a number of sera which formed soluble complexes of antigen and antibody in the zone of excess antibody. With these sera an equivalence zone could be determined in which precipitin tests detected neither antigen nor antibody in the supernatants. When the same supernatants were assayed for antibody by agglutination of tanned erythrocytes sensitised with antigen and for antigen by tests of enzyme activity, the co-existence of enzyme and antibody activity was observed. Some sera, however, gave supernatants in the equivalence zone, in which neither antigen nor antibody could be detected by any of these tests; such sera could be employed (15) to determine the combining ratio of antigen and antibody directly in the zone of excess antibody and indirectly (16) in the zone of slight antigen excess.

So far investigations have been considered in which catalytic properties were used as markers for the detection of enzyme as an antigen. In the following sections the kinetic interaction of substrate, enzyme, and antibody will be reviewed.

Antibodies as Enzyme Inhibitors

Enzymes, like other proteins, are antigenic. Their catalytic action is usually reduced by the corresponding antibody. A plot of residual enzyme activity, as a function of the quantity of antibody added to a constant amount of enzyme (neutralisation curve),[4] shows first a linear decrease, then

[4] For a discussion of the advantages of this technique over one in which antibody is kept constant see (17).

a deviation from linearity and finally, in extreme antibody excess, a residual level of activity which is either not at all or only slightly influenced by further addition of antibody. Complete inhibition is a limiting case of this state of affairs, depending as much on the sensitivity and duration (17 to 20) of the enzyme assay as on the properties of the enzyme-antibody complex. Residual enzyme activities observed with sera obtained by only one technique of immunisation, should not be considered as absolute values since the degree of inhibition appears to depend on the conditions of immunisation and may be influenced by genetic factors as is the quantitative response (21) and the nature of isoagglutinins (22).

A continuous increase in inhibiting power of successive bleedings obtained from rabbits in the course of prolonged immunisation with ribonuclease has been observed, sera from early bleedings inhibiting 60 to 70 per cent and from late bleedings 96 to 98 per cent of the enzyme activity (23). A comparison of the results reported by Gessard in 1901 (24) and in 1902 (25) indicates that this evolution of antibody properties may also occur when tyrosinase is the antigen [see also (20 and 26)] and that a general phenomenon, the strength of the binding between antibody and antigen ("avidity"), may be involved (27, 28, 29).

Contradictory results obtained by different workers with the same antigen (catalase and ribonuclease) may find their explanation in such variations of antibody properties.

Marucci & Mayer (30) have shown the relation between the neutralisation of urease and the number of antibody molecules combined with each antigen molecule: 12.3 molecules of antibody caused 41 per cent and 22.5 molecules 78.5 per cent inhibition. Data are, therefore, only comparable when they have been obtained in a corresponding range of antibody excess. The discrepancy in the degree of inhibition observed with ribonuclease (23, 31) may be due not only to differences in immunisation but to the fact that one of the observations was made in slight and the other in extreme antibody excess.

Since the qualitative changes of antibody properties in the course of prolonged immunisation are gradual rather than abrupt (23, 32), a given serum contains a mixture of antibodies of varying properties. The antibody fraction which combines most readily with the antigen (11) probably contains the antibodies with the highest inhibiting power.

A survey of the data concerning the inhibition of enzymes by excess antibody, some of which are collected in Table I, indicates that complete absence of inhibition by antibody is rare and confined to systems in which the substrate has a low molecular weight.

The velocity of combination of enzyme and antibody depends on the concentration of these two reactants, on the combining power of the antibody (40), and on temperature (35, 40). Changes of pH between 6.6 and 8.2 seem to have no effect on the activity of lecithinase combination with antibody (68). Judging by the interaction between urease and antibody (30), complement does not contribute to the inhibitory power of antibody.

TABLE I

DEGREE OF INHIBITION BY EXCESS ANTIBODY AND MOLECULAR WEIGHT OF SUBSTRATE*

Enzyme	Substrate and Molecular Weight (M)	Degree of Inhibition (%)	Reference
Catalase (beef liver)	Hydrogen peroxide (34)	6-3 (Precipitate 27–68)	(33)
		none	(34)
		35–60	(35)
Urease (jack bean)	Urea (60)	66, 78.5	(36, 30)
Carboxylase (yeast)	Pyruvic acid (80)	100	(37)
Tyrosinase (*Psalliota campestris*)	Tyrosine (181)	None; ~100	(38, 20; 39)
(*Neurospora*)	Tyrosine (181)	None	(104)
(*Glomerella cingulata*)	Tyrosine (181)	50–80	(20)
(*Russula*)	Tyrosine (181)	100	(25)
Tryptophanase (*Bact. coli*)	Tryptophan (204) Pyridoxal phosphate (247)	100	(41, 42)
α-glycerophosphatase (yeast)	Ca-α-glycerophosphate (210)	100	(19)
Lipase (*Mycobacterium phlei*)	Tributyrate glycerol (302)	100	(43)
Phosphoglucose Isomerase (*Schistosoma mansoni*)	fructose-6-phosphate (244)	⩾ 53	(53)
Penicillinase (*Bacillus cereus*) Strain 569	Penicillin (254–366)	98–100	(45, 11)
Strain 5/H	Penicillin (254–366)	40	(11)
β-Galactosidase (*E. coli*)	Lactose (350)	None	(7)
Carboxypeptidase (bovine)	Carbobenzoxyglycyl-L-tryptophan (365)	⩾ 70	(40)
Hexokinase (yeast)	Glucose (180) ATP (507)	100	(44)
D-Glyceraldehyde-3-phosphate dehydrogenase (yeast)	DPN (691) 3-phosphoglyceralde- (154)	90–95	(46)
Lactic dehydrogenase (rat muscle)	DPNH (693) Pyruvic acid (80)	45–75	(47)
(rabbit muscle)	DPNH (693) Pyruvic acid (80)	⩾ 60	(48)
(*Schistosoma mansoni*)	DPNH (693) Pyruvic acid (80)	66	(49)

* Based on a table in (18); † phospho ester link; ‡ fatty acid ester link.

TABLE I (*continued*)

Enzyme	Substrate and Molecular Weight (M)	Degree of Inhibition (%)	Reference
Papain (*Carica papaya*)	Gelatin	71.5–72.6; 100	(50, 51)
	Casein	58–72.5	(50)
Gelatinase (*Clostridium tetani*)	Gelatin	100	(52)
Proteinase (group A type 3 haemolytic streptococcus)	Protein extract from muscle; diazotised skin proteins	100	(54)
Collagenase (*Clostridium welchii*)	Collagen	100	(55)
Tropomyosinase (η antigen; *Clostridium oedematiens*)	Tropomyosin	~100	(106)
D-Ribonuclease (beef pancreas)	Ribonucleic acid	20–30, up to 98	(31, 23)
(*Bothrops jararaca*)	Ribonucleic acid	100	(56)
Deoxyribonuclease (*streptococcus*)	Deoxyribonucleic acid	100	(57)
(beef pancreas)	Deoxyribonucleic acid	~100	(58)
(*B. jararaca*)	Deoxyribonucleic acid	$\gtrdot$ 87	(59)
Hyaluronidase (*C. welchii*)	Mucoprotein (umbilical cord)	100	(60)
(bee)	Mucoprotein (umbilical cord)	~100	(61)
Amylase (barley)	Starch	95	(62)
Carbohydrase (*B. palustris*)	*Pneumococcus* Type III, Type VIII polysaccharides	~100	(63)
Lysozyme	*Micrococcus lysodeikticus*	100	(64, 65, 66)
Lecithinase† (*C. welchii*)	Lecithin (emulsion)	100	(67, 68, 17)
Lecithinase‡ (*Crotalus terrificus*)	Lecithin (emulsion)	100	(68)
(bee)	Egg yolk	$\gtrdot$ 80	(61)

The high dilutions in which the interaction between enzyme and antibody can be studied have allowed changes in the degree of inhibition to be examined during the course of incubation; nevertheless the reaction has been found to be essentially complete in 1 min. and complete in 30 min. (68), or to be almost instantaneous (35).

Apart from this fast primary combination a secondary phase has been described; at high absolute concentrations of *Clostridium welchii* lecithinase and its antibody (from horse), a gradual decrease of the small residual en-

zyme activity could be observed after flocculation had taken place. This secondary decrease of activity during prolonged incubation was apparently due to a change in the structure of the precipitated antigen-antibody complex (17).

So far the residual activity of enzyme-antibody mixtures has been considered as it appears at one substrate concentration and at a constant time after the addition of substrate. The effect of time and substrate concentration on this activity has been the subject of several investigations. The residual activity of an incubated mixture of pyruvic acid reductase (rat muscle) and its antibody was shown to depend on the concentration of the substrate pyruvate (47). A similar dependence was observed between lactic dehydrogenase of *Schistosoma mansoni* antibody (from a rooster) and DPNH (49). In other enzyme-antibody systems this relation between residual activity and substrate concentration was not observed. Incubated mixtures of *C. welchii* lecithinase and antibody had the same relative activity over a considerable range of substrate concentrations (18). A Lineweaver & Burk plot (69) of reciprocal enzyme activity as a function of reciprocal substrate concentration showed the inhibition to be noncompetitive. The reaction did not conform to the simple kinetics of reversible reactions between enzyme, antibody, and substrate. A plot of

$$\log \left\{ \frac{v}{v_A} - 1 \right\}$$

against the logarithm of the antibody volume did not give the straight line expected from the equation

$$\log \left\{ \frac{v}{v_A} - 1 \right\} = \log \frac{1}{K_A} + r \log A \cdots \tag{1}$$

where

v = velocity of enzyme reaction
v_A = velocity of enzyme reaction in the presence of antibody
A = concentration of antibody
K_A = dissociation constant of the enzyme-antibody complex
r = molecules of antibody combined with one molecule of enzyme or one enzyme site.

In another noncompetitive system, carboxypeptidase and antibody, strict conformity to the kinetics of noncompetitive reactions was observed [Smith *et al.* (40)]. The value (r) in equation (1) was found to be one between 25° and 39.5° and two at 6°C.

With carboxypeptidase as with tryptophanase the preincubated complex of antigen and antibody was found to dissociate on dilution (40, 42).

Dissociation of enzyme-antibody complex in the presence of substrate was first demonstrated in a cross-reacting system between the antibody to *C. welchii* lecithinase and the lecithinase of *Clostridium bifermentans*. It was later also observed in the interaction of the homologous antibody with *C. welchii* lecithinase but was weak and could only be demonstrated with high-

concentrations of reactants near the region of antibody excess (17, 18). Dissociation occurred also in mixtures of α-glycerophosphatase and anti-phosphatase (19), and as in the cross-reaction already mentioned (18), was observed in antigen as well as in antibody excess. In the phosphatase system the extent of the dissociation depended on the substrate concentration. The secondary slow decrease of residual activity which accompanies precipitation of lecithinase-antibody mixtures has already been mentioned. Dissociation was observed in the same experimental conditions, and in no others, and was little affected by the progressive changes in residual activity (17). It is likely that the dissociation may reflect a reversal in the state of the aggregates and that it may be due to equilibrium between floccules and less highly aggregated and soluble complexes of antigen and antibody.

A few general features arise from a summary of the diverse observations so far mentioned. The degree of inhibition of an enzyme increases with the number of antibody molecules combined. The inhibiting power of immune sera of rabbits varies in the course of immunisation. Enzymes acting on macromolecules are, as a rule, completely inhibited by their antibodies. Antibodies which precipitate an enzyme, but do not inhibit it, have only been observed with enzymes acting on substrates of low molecular weight (see Table I). The degree of inhibition has been shown in a few enzyme-antibody systems to be dependent on and in others to be independent of the concentration of substrate.

Once enzyme has combined with antibody, the combination may become irreversible, and the enzyme may act on substrate only through catalytic sites not blocked by the antibody. Equilibrium is established with greater certainty when antigen, antibody and substrate are added simultaneously.

The Simultaneous Addition of Substrate, Antibody, and Enzyme

The activity of enzyme after incubation with antibody depends on the quality of the antibody determined by the state of immunisation of the antibody donor, the relative quantity of antibody, the period of incubation and, in some instances, on the concentration of substrate. The relation between the degree of inhibition and the period of incubation has not been studied in any detail, primarily because incubation periods of seconds or fractions of seconds and temperatures below 10°C. would be required to allow such observations to be made (40).

Antibody is a less effective inhibitor when combining with enzyme in the presence rather than in the absence of substrate; either in the simultaneous addition of enzyme-antibody and substrate and in the addition of antibody to a mixture of enzyme and substrate. This "protection of enzyme by substrate" has been demonstrated for amylase (62), penicillinase (45), lecithinase (18, 68), carboxypeptidase (40) and tyrosinase (20) and represents, therefore, one of the more safely established facts in the field. The one exception to this general behaviour (48) will be discussed in a later section.

The degree of inhibition caused by a dose of antibody under conditions

of simultaneous addition seems to be essentially independent of the substrate concentration for *C. welchii* lecithinase and bovine carboxypeptidase. Both these enzymes obey the equation (1) for noncompetitive reactions and r, the number of inhibitor molecules per enzyme site, is equal to one (18, 40) but, surprisingly, is equal to two for the interaction between carboxypeptidase and antibody at 6°C. (40).

An increase in concentration of the enzyme activator, that is, of calcium, favoured the combination between *C. welchii* lecithinase and substrate at the expense of combination between enzyme and antibody. As a consequence, the neutralisation curve of lecithinase was shifted towards higher concentrations of antitoxin (18). An apparently contradictory picture has thus emerged; the difference between incubation and simultaneous addition points towards the competitive nature of inhibition by antibody, as does the effect of Ca concentration in the lecithinase system. On the other hand, a noncompetitive relation between antibody and substrate was observed when the substrate concentration was varied.

In recent studies a marked effect of the substrate was observed when the antibody was added to enzyme and substrate in two cases, and to enzyme and coenzyme, in another. The inhibitory effect on phosphoglucose isomerase (*S. mansoni*) of serum from an immunised rooster was reduced when the enzyme was incubated before the addition of the antiserum either with fructose 6-phosphate or with glucose 6-phosphate ($M=244$). When the concentration of fructose 6-phosphate was doubled, the inhibition which was 47 per cent in the absence of substrate, changed from 30 to 14 per cent (53). A similar protection of tyrosinase (*Glomerella*) has been described [Owen & Markert (20)]. The inhibition of lactic dehydrogenase (*S. mansoni*) by antiserum was markedly reduced by low concentrations of DPNH and completely abolished by high concentrations (49).

It remains to be seen whether the apparent differences with different enzymes can be unified and whether internal contradictions, as in the case of lecithinase, can be resolved experimentally. These differences and contradictions may be due to the application of concepts, developed for inhibitors which are univalent and very small in relation to the enzyme to bivalent macromolecular inhibitors.

Simultaneous Addition of Apoenzyme, Antibody, Coenzyme, and Substrate

We have seen that the combination of enzyme and antibody is less complete when occurring in the presence of substrate than it is in experiments in which enzyme and antibody are incubated before addition to substrate. There is so far only one exception to this rule, so that it might be used with some confidence to deduce the sequence of attachment in reactions between coenzyme (CoE), substrate (S) and apoenzyme (A). Three initiating reactions can be visualised [modified from (17)] and the effect of CoE and substrate on enzyme-antibody interaction would depend on the pair of components initiating the reaction.

Table II shows these possibilities and the expected influence of (CoE) or (S) on the inhibitory power of antibody.

This scheme must remain tentative until several cases have been studied of simultaneous addition with enzymes which do not require coenzymes and act on substrates of low molecular weight (M = 50 to 100). There are reasons for believing (see final section) that a substrate of low molecular weight may not interfere with inhibition as effectively as a substrate of high molecular weight. Until this has been decided experimentally we must consider the possibility that small substrate molecules may not "protect" the enzyme.

TABLE II

EFFECT ON NEUTRALIZATION CURVE

Initiating combination	Simultaneous addition of antibody, apoenzyme and	
	(S)	(CoE)
1) (A)(CoE) combine and then act on (S)	−	+
2) (A)(S) combine and then act on (CoE)	+	−
3) Either (A)(CoE) acting on (S) or (CoE) acting on (A)(S) can initiate the reaction	+	+
4) (CoE)(S) must interact before (A) can participate in the reaction	−	−

+ protects enzyme. − does not protect enzyme.

The first observation to which the scheme proposed in Table II can be applied was that of Krebs & Najjar (46) who found that DPN reduced the inhibiting power of an antiserum to d-glyceraldehyde 3-phosphate dehydrogenase, when it was added at the same time as enzyme and antibody, whereas the substrate, d-glyceraldehyde 3-phosphate, did not interfere with the action of the antibody. It could thus be inferred (Table II) that combination of DPN and apoenzyme represents the primary step in the enzyme action. The reaction mechanism proposed by Racker & Krimsky (70) would agree and that proposed by Segal & Boyer (71) could coexist with this scheme. There is good evidence for the existence of a compound between this enzyme and DPN; the two components can only be separated by fairly drastic methods, and they crystallise together (72).

Since then a similar effect has been described for the lactic dehydrogenase of *S. mansoni* [Henion, Mansour & Bueding (49)]. Simultaneous addition of enzyme, antibody, and pyruvate did not reduce the inhibitory effect of the immune serum whilst DPNH in low concentration reduced the inhibitory effect and in high concentration abolished it.

There is, however, one report describing a combination between enzyme and antibody which is unaffected by substrate or coenzyme (Table II); it

is of the action of antibody on lactic dehydrogenase from rabbit muscle [Mansour, Bueding & Stavitsky (48)]. Preincubation of the enzyme with pyruvate, lactate, DPNH, or DPN did not affect the inhibitory action of the immune serum. The reaction is unusual in that a decrease in the concentration of either the coenzyme or the substrate resulted in increased and not in decreased activity of the enzyme-antibody mixture. This result could be accounted for by the need of an initiating interaction between coenzyme and substrate prior to any participation of apoenzyme (4 in Table II). This would imply that the simultaneous addition of all the components, apoenzyme, coenzyme, substrate, and antibody, would lead to interference with the inhibitory effect of antibody, a prediction which could be tested experimentally.

Cross Reactions

It has been suggested that enzymes cross-react by virtue of identical catalytic sites. This would imply that the catalytic site is identical with the site of antibody combination; cross reactions between enzymes are being reviewed to examine this proposition.

Model experiments by Landsteiner (73) have shown that the attachment of polypeptides to proteins through diazobenzene confers a new specificity. In pentapeptides the terminal amino acid contributed decisively, but not exclusively, to the new specificity since even the amino acid most distant from the end of the peptide chain was of importance. By absorption with structurally related peptides an antibody fraction could be obtained which reacted only with the homologous polypeptide. Landsteiner was well aware that much larger polypeptides would be required to gain information on "the size and nature of the determinant groups in proteins" (74). The direct addition of polypeptides by the initiation of the polymerisation of *N*-carboxyamino acid anhydrides by protein may open the way to such studies (75). It now appears likely that terminal amino acids do not play a very important part in determining specificity of proteins. Bovine serum albumin from which 5 to 6 residues have been removed from the C-terminus cannot be distinguished by the quantitative precipitin test from native bovine albumin [White, Shields & Robbins (76)].

Experiments with protein antigens with two different synthetic determinants (90, 91, 92) have recently (92) furnished evidence of a single combining site on some antibody molecules being adapted to both determinants. This led Francis, Mulligan & Wormall (92) to the suggestion that antibody receptor areas are complementary to a relatively large area of the surface of the antigen and that the antibody molecules (within a single serum) may vary considerably in their adaptation to the homologous antigen. Hence some fractions of antibody molecules may be well adapted to only one of the two chemical determinants introduced into an antigen.

Presumptive evidence for antibodies being adapted to large areas on the antigen has also been obtained by studies on acquired immunological toler-

ance (107) to human albumin. Rabbits injected with human albumin at birth fail to form antibodies to this antigen in later life. The majority of such animals also fail to respond to diazo-human albumin. Two animals were found to respond to injections of diazo-human albumin with the formation of antibodies which were primarily adapted to the diazo group but possessed, nevertheless, some power to react with human albumin (109). Antibody could thus not adapt itself to the small diazo group alone, but had also to adapt to the area surrounding the chemical determinant.

The conclusion of Francis, Mulligan & Wormall seems to hold also for determinants which consist of the amino acid sequences of a native protein. Lapresle (100) has demonstrated this in studies of the action of a proteolytic enzyme on a pure protein and of the immunological behaviour of the resulting products. Human albumin was digested by a peptidase obtained from rabbit lymph. Before the digestion, the human albumin had given only a single precipitin zone as judged by electrophoresis in agar followed by reaction with immune serum to the native protein. The digest antigen, on the other hand, reacted with the same serum in three distinct and independent precipitin zones corresponding to three different electrophoretic mobilities. The antigen had been broken into components which reacted as independent antigens. This could only occur if different groups of antibody molecules were adapted to distinct parts of the surface which were separated by enzyme action from the intact antigen molecule.

So far, we can conclude that fairly large areas of the antigen surface act as immunological determinants, that a serum contains antibodies adapted to different areas of the surface and that the end groups of the protein do not play an especially important role.

Functionally similar proteins of related species are usually antigenically similar; the degree of cross reaction between them is greater the closer the taxonomic relationship (77, 78, 79). This relationship exists between functionally similar proteins of different species and generally not between functionally different proteins of the same species.[5] It is to be expected that this should apply to proteins which have enzyme activity as much as to any other protein. The absence or presence of cross reaction does not, therefore, permit any inference about the relation between the antigenic determinant and the site of enzyme activity.

Cross reactions have never been observed between functionally similar enzymes from mammals and those from either bacteria (57), yeast (44, 46, 103), or helminths (48, 49, 53), or between an enzyme from cuttle fish and one from mushroom (80, 81).

The absence of a cross reaction between the tyrosinases of *Glomerella* and *Neurospora* (82, 83) and between those of *Psalliota campestris* and

[5] Recently an exception to this, a cross reaction between two constituents of different electrophoretic mobility but from the same serum, was reported by Williams & Grabar (102).

Lactarius piperatus (38) may appear surprising. However, isodynamic enzymes are not necessarily of the same biochemical origin. The respiratoyr enzymes: haemoglobin, haemocyanin, and haemerythrin are an instance of enzymes with entirely different chemical composition, performing the same function (101). Even two enzymes of similar catalytic specificity and synthesised by the same organism (cell-bound and exocellular penicillinase of *B. cereus*) may fail to cross-react (12).

There are frequent reports on cross reactions between functionally similar enzymes from closely related organisms: mammals (33, 48), plants (84), bacteria (45, 85 to 88), snakes (59) and worms (49).

In short, cross reactions between enzymes do not depend on catalytic but on taxonomic factors. The relation of host and parasite (rabbit or rat and schistosome) does not seem to be an exception to this rule (48, 49, 53), though an extension of these studies to viruses could be of value.

Even with very closely related antigens, such as Gz (β-galactosidase) and Pz of *E. coli* (8), there was a small portion of antibody which combined only with the homologous antigen, and a larger portion which combined preferentially with homologous antigen. There were no antibody molecules which combined as firmly with heterologous as with homologous antibody. The homologous antigen Gz displaced Pz from soluble complexes formed in antigen excess but not from washed preformed precipitates [(8); cf. also Mayer & Heidelberger (89)]; the degree of reversibility of the union between antigen and antibody having thus been greatly reduced in the process of precipitation.

The cross reaction between the lecithinase of *C. welchii* and *C. bifermentans* has been examined in some detail. The quantity of antibody required to inhibit the activity of the heterologous enzyme was much greater than that required to inhibit the homologous enzyme. The ratio of these quantities differed widely when a number of sera were compared. Each enzyme was precipitated by its homologous but not by its heterologous antibody (85, 86). In the cross reaction between lecithinase of *C. bifermentans* and the antibody to the lecithinase of *C. welchii* a slow but steady dissociation of the antigen-antibody complex was observed. The difference between inhibition by antibody added to enzyme before addition to substrate and added to enzyme at the same time as substrate was greater than in the homologous system (18). Even in high concentration the antibody was not as effective an inhibitor of the heterologous as of the homologous enzyme.

In a comparison of two very closely related enzymes, the exocellular penicillinases of two strains (5/B and 569) of *B. cereus*, a similar relationship was detected by partial absorption of the antibody (11); 569 antibody gave a 40 per cent higher neutralisation titre against 569 than against 5/B enzyme as judged by the initial linear portion of the neutralisation curve. When part of the antibody was removed either with 569 or 5/B, the difference in neutralisation titre became more marked; it was 160 per cent and 300 per cent higher for the homologous enzyme when 70 per cent and 90 per cent,

respectively, of the homologous antibody were removed. Furthermore, 569 antibody, absorbed with 5/B enzyme, inhibited 5/B much less effectively than 569 enzyme and failed to precipitate 5/B enzyme.

These results correspond very closely to findings with antigens into which two different chemical determinants have been introduced (92), and the interpretation of these (92) can be applied to the enzyme experiments—the antibody best adapted to the homologous antigen would be most readily precipitated; badly adapted ('nonavid') antibody is unable to precipitate unless coprecipitated by well-adapted antibody.

Enzymes of similar catalytic specificity which are antigenically unrelated and others which are very closely related have been mentioned. There is a third group to be considered consisting of enzymes with similar catalytic function which are known to be different in terms of some chemical or physico-chemical property (93 to 97) but which are of a common biochemical origin. Of these ribonuclease A and B have been compared by several immunological methods and found identical. With some but not all sera a small difference was found by comparing their quantitative precipitin curves in the zone of antigen excess (15).

Finally, the identity of enzymes has been established by immunological methods for the lecithinases of *Clostridium haemolyticum* and the β-toxin of *Clostridium oedematiens* (87), for mutants of *Glomerella* (82) and for a fraction of the cell-bound (β) and exocellular penicillinase of *B. cereus* (12). The method of partial absorption, described by Pollock (11) should provide a valuable tool in such studies.

The nature of cross reaction is probably best revealed by a comparison of antigens which show considerable immunological differences. From such studies, it would appear that only some of the antibody molecules adapted to a homologous antigen can react with a heterologous antigen. Even the antibody molecules capable of combining to form relatively soluble compounds with heterologous antigens, are relatively inefficient inhibitors in extreme antibody excess and are less effective inhibitors in simultaneous addition than after preincubation.

There is, so far, no evidence to indicate that cross reaction between enzymes depends on the identity of catalytic sites.

Mechanism of Enzyme Inhibition by Antibody

It is obvious that antibody adapted to the catalytic site of an enzyme wili inhibit the enzyme. Since it is not equally evident that adaptation to the catalytic site is a necessary condition of the inhibition by antibody, thls aspect will be discussed.

Two types of interactions between enzyme and antibody have been mentioned in the preceding pages, competitive and noncompetitive. These can be defined[6] in terms of the reactions governing combination between

[6] For a brief summary of this field see (99).

enzyme (E), substrate (S), and inhibitor (I):

for competitive inhibitors: $E + S \rightleftharpoons ES$, $E + I \rightleftharpoons EI$

for noncompetitive inhibitors: $E + S \rightleftharpoons ES$, $E + I \rightleftharpoons EI$ and $ES + I \rightleftharpoons ESI$

Hence, inhibition by noncompetitive inhibitors depends only on the concentration of the inhibitor while inhibition by competitive inhibitors depends on the relative concentration of enzyme and substrate.

These concepts were developed in terms of inhibitors and substrates of a molecular weight very similar to one another and considerably smaller than the enzyme. For this reason it was correct to conclude that an inhibitor which, by kinetic criteria, competed with substrate could do this only by combining with the same site of the enzyme as did the substrate. This topographic interpretation loses its validity when the inhibitor molecule is as large as, or larger, than the enzyme and when the inhibitor is known to combine with enzyme over a large area and to be well-adapted to part or all of this combining area. Such an inhibitor can clearly act kinetically as a competitive inhibitor and may nevertheless not be especially well adapted to the relatively small area of catalytic action. It follows, therefore, that competitive inhibition by antibodies must be interpreted as a kinetic phenomenon only and not in terms of combination between antibody and the site of enzyme action. Kinetic studies are therefore unlikely to provide information on the identity of catalytic centre and antibody combining centre. It remains to examine evidence that antibody inhibits without combining with the site of enzyme action.

An enzymically inactive precipitate of *C. welchii* lecithinase and antibody was found to remove antibody from a solution of immune serum. In other words, after the addition and subsequent removal of the precipitate the inhibitory power of the lecithinase antiserum was found to be lowered. The antibody had combined in the precipitate with enzyme molecules which were already inhibited and these antibody molecules were nevertheless able to inhibit enzymes. This was demonstrated by the decrease in potency of the serum from which they had been removed (17).

The activity of urease-antibody precipitates decreases with increasing ratio of antibody to enzyme molecules [Marucci & Mayer (30)]. This could be the outcome of random combination with antibody either near or far from the catalytic centres. The probability, that catalytic centres are covered by antibodies not specially adapted to them, would increase as the surface of the enzyme becomes more completely covered by antibody.

Two enzyme systems have been described in which substrate did not interfere with inhibition as judged by increasing substrate concentration but seemed to affect the combination of antigen and antibody in simultaneous addition. In the case of lecithinase this effect could be shown to be at least partly due to the slow equilibration of the enzyme-antibody complex with substrate. The question arose, why this equilibration should be so slow, and why the combination of enzyme and antibody was so strongly influenced

by the presence of substrate when a gradual increase in the concentration of substrate had so little effect. It seemed that the aggregation of antigen-antibody complexes might be responsible for this phenomenon [17, 18; see also (4)]. Aggregation between antigen-antibody monomers is known to occur almost immediately after antigen and antibody have combined. The presence of substrate would not only tend to reduce the size of particles of the aggregate (17) but possibly also to influence aggregate organisation. The resulting precipitates would have a considerable portion of their surface composed of antigen. A suggestion that this may be the case has come from the work of Krebs & Najjar (46) who found higher enzymic activities in precipitates formed in the presence than in those formed in the absence of substrate.

Cohn & Torriani (8) found a homologous protein antigen able to displace a heterologous antigen from its combination with antibody, as long as the antigen-antibody complex was in solution, but unable to do so once precipitation had occurred. This would suggest that aggregation reduces the reversibility of combined antigen and antibody and that this reduction is most marked when aggregation is complete, that is, in the precipitate.

Existing information does not allow one to distinguish between the relative contributions to steric hindrance made by the basic combination of antibody and antigen and by the aggregation of this primary compound. The relative importance of either of these mechanisms, but particularly of aggregation, would depend on the molecular weight of the substrate molecule. This might explain why failure to inhibit has so far only been described for antibodies to enzymes acting on small substrates.

On the basis of experiments with alcohol dehydrogenase, it has been proposed that antibodies formed during prolonged immunisation are not adapted to the antigen but to the complexes of antibody and antigen (98). It is clear that such antibodies could increase the inhibitory effect of aggregation by stabilising the antigen-antibody complex or its polymer.

Inhibition will necessarily appear to be noncompetitive when the affinity between inhibitor and enzyme is very great compared to that between substrate and enzyme (see 110, 111). The affinity of antibodies for antigen varies from serum to serum [p. 374; see also (20)]. It is thus possible that observed differences between different systems of enzyme, antibody and substrate may not be due entirely to antigenic or to kinetic differences between enzymes but that they may be due to differences in the technique of immunisation and in the resulting antibody. It will be necessary to consider and to analyse the inhibitory action of antibodies formed in response to the same antigen, but by different techniques of immunisation, before the mechanism by which antibody inhibits can be fully understood and before the interaction of enzyme and antibody can be employed as a reliable tool for the study of enzyme properties.

Conclusions

It is obvious that the kinetic aspects of the interaction between enzyme, antibody, and substrate need further fundamental work. The protection of enzyme against its antibody by substrate (p. 378) may be analogous to that of toxin by tissue receptor and may explain why antitoxin is ineffective after the toxin has reached its point of attack. Furthermore, the protection of apoenzyme by coenzyme may provide a tool for studies of the interaction between enzyme and substrate. The analysis of such problems requires an understanding of the nature of inhibition by antibodies. Three mechanisms have been considered for this: (*a*) adaptation to the catalytic site; (*b*) adaptation to a site other than the catalytic centre but near to it and consequent steric hindrance; (*c*) aggregation of the antigen-antibody complex and steric hindrance by the structure of the aggregate.

The first of these three mechanisms would permit only a small fraction of antibodies to be inhibitory; if every antibody molecule were inhibitory the valency of the antigen would be identical to the number of catalytic sites on the enzyme molecule. The second mechanism would allow the majority, but not all antibody molecules, to be inhibitory, at any rate when the enzyme had only one catalytic site. The third mechanism would lead to every antibody molecule being inhibitory if it can participate in aggregation.

These three mechanisms are, of course, not mutually exclusive; they may, and probably do, coexist, their relative importance depending on the size of the substrate molecules. The problem may be advanced by more data on the variation in the inhibitory properties of antibody as a function of the pathway and duration of immunisation. With a full understanding of the mechanism of inhibition a powerful tool will become available to dynamic biochemistry and to dynamic immunochemistry.

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BACTERIAL GENETICS[1,2]

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Microbial genetics is still in a phase of expansion and the splitting of the subject this year has somewhat relieved the reviewer's labours. The present paper attempts to cover the developments in the genetics of bacteria which have taken place in the years 1955 and 1956, i.e., subsequent to the publication of the previous review by Zelle (181). Work on phage will be considered only insofar as directly relevant. The author is also grateful to the editors for their encouragement in suggesting that attention be focussed on selected problems. The choice of subjects to which more space and time has been dedicated is inevitably dependent on the author's taste and background. Although a general picture has been attempted, omissions may have occurred, voluntarily or involuntarily. Moreover, the tyranny of space has made it impossible to consider cytology, radiation, and biochemical genetics, except for a few selected themes. Apologies are offered in advance to readers and writers for these omissions.

The following topics will be discussed: mechanisms of genetic transfer; genetic changes in individuals and populations; phenogenetics; some cytological contributions; taxonomy and the species problem.

GENETIC TRANSFER

A great deal of work has been devoted to the problem of genetic exchange. We can distinguish at least three modes of genetic transfer in bacteria, and undoubtedly finer distinctions will have to be made in the near future. First, deoxyribonucleic acid can carry genetic information from a donor cell to a recipient into which it is incorporated without any known intermediary in the transfer. Second, phage can act as a carrier of genetic information from a donor in which it has developed, to a recipient susceptible to it. Third, conjugation can occur between two cells with exchange of nuclear material; this is now established in *Escherichia coli* strains, and is postulated in all other cases in which cell to cell contact is known to be necessary for genetic transfer. Examples of conjugation have been described in which the transfer clearly takes place in one way only, so that one can distinguish a donor and a recipient; the former acts as a male gamete and the latter as a female gamete.

Clearly, these various mechanisms are separate steps or paths of one evolutionary process leading to sex or starting from sex, as defined on the

[1] The survey of the literature pertaining to this review was completed in December, 1956.

[2] The following abbreviations will be used: DNA (Deoxyribonucleic acid); F− (Female or receptor strains of *E. coli*); F+, Hfr (Male or donor strains of *E. coli*); HFT (High frequency of transduction); TP (Transforming particle).

basis of observations on species of a higher level of organization. In higher forms, sex is accompanied by the existence of specialised organs, specialised cells and processes, and chromosome cycles which have varying degrees of complication and exactitude. Specialization and meticulousness of detail have weakened or disappeared among bacteria, or have never appeared, at least as far as we know. But the main biological consequence of sex, i.e., recombination or the formation of new genotypes by a reshuffling of the hereditary characteristics of the parents, occurs nonetheless. It must play the same role, namely, that of supplying a basis for the selection of fitter genotypes in a changing environment, more efficiently than does mutation alone. Obviously, the third type of mechanism listed as operative in bacteria is nearest to what is called sex in higher organisms, and more nearly deserves the name of sexual transfer.

It may be noted that, among some organisms, two types of recombination can be found, meiotic and mitotic. The latter occurs in the "parasexual" cycle (136) in which recombination in the progeny of a diploid nucleus is not, or not necessarily, accompanied by reduction to the haploid state. This distinction retains full meaning, especially when both types of recombination can be shown to occur in the same organism. In bacteria the situation is somewhat unsettled, and in some cases the wildest hypotheses might still be permissible (113), but for Occam's razor. Hints of sexual differentiation at the cellular level are available, and reduction after conjugation in *E. coli* K-12 may be fairly regular, in agreement with a truly sexual cycle. On the other hand, the structure of bacterial chromosomes is unknown, and the existence of centromeres in particular is entirely hypothetical. Hence it is possible that the terms "mitosis" and "meiosis" cannot be rigorously applied to the unknown processes of reproduction of bacterial chromosomes. When fragmentary hybridation is the rule, as is probably true of most types of genetic transfer in bacteria, no precise meiotic mechanism is necessary. A fragment has a sufficiently high chance of being eliminated spontaneously without specialised mechanisms, and the cell can be relieved of the burden of maintaining specialised organs for the purpose of reduction to the haploid condition.

Mostly speculative also is the state of our knowledge concerning another important aspect, the mode of genetic exchange at the chromosome level. The problem can be concisely stated as follows: is there actual incorporation of the fragment into the progeny, or is it only the genetic information contained in the fragment which is incorporated, by copy? Various models have been offered. Clear evidence exists only on one part of this problem, i.e., whether the incorporation is partial or complete. The evidence favours partial incorporation (39, 79), strongly suggesting the analogy with crossing over in higher organisms. Since, in higher organisms, one problem, entirely akin to the above one, is still open, the analogy does not advance us too much. Several discussions on the matter will be found in various papers of the excellent Symposium on Genetic Recombination, held at Oak Ridge in 1954.

Some light is being shed on the problem by work on duplication of phage chromosomes, using P^{32} as a marker. Results of a peculiar method of autoradiography tend to show that the parent chromosome structure is unaltered by replication, i.e., does not break down in smaller units; at least such is the behaviour of a major fraction of phage DNA (110). Genetic exchange should therefore be the outcome of an alternating choice in the copy from two or more parental models, in agreement with recent genetic work (16).

On the other hand, a different approach, also using P^{32} (157, 158, 159), suggests the possibility that DNA duplication may not be a direct event, but rather one which requires an intermediate to which genetic information is transferred *pro tempore* (158).

Heterokaryosis.—The reproduction of mycelia carrying nuclei of different origins has been demonstrated in *Streptomyces*, using strains marked by auxotrophic mutations. In the case of *Streptomyces coelicolor*, heterokaryosis is a stage preliminary to nuclear fusion and recombination (150). In *S. griseus* recombination has not been detected (13). Hyphal fusion, from which presumably heterokaryosis originates, has been confirmed cytologically (43). The heterokaryotic condition can be transmitted through hyphal fragments, but is destroyed in the spores which are uninucleate (13).

The shape and mode of reproduction of bacillary and small coccoid forms makes it unlikely that heterokaryosis could be more than a rapidly transient phenomenon in these forms.

New situations encountered in fragmentary hybridation have demanded new terminology (126, 177). Censors (45) of the early 1953's will undoubtedly have been appalled at the wealth of new terms! The term *transduction* has come into general use. First applied to phage mediated transfer, its original definition practically coincides with fragmentary hybridation (101) and therefore includes "transformation" as well as possibly some cases of transfer by cell-to-cell contact.

General reviews on the subject of genetic transfer: (23, 99 to 101, 141).

Naked, or DNA-Transduction (Transformation)

Nature and size of genetic fragments.—Further confirmation that transforming principles (TPs) consist of deoxyribonucleic acid has been obtained by work on purification and attempted fractionation with chromatographic techniques (9, 46, 108). The transforming activity of deoxyribonucleic acids has been slightly enriched. Undoubtedly this promises to be a fascinating field for future work. The size of transforming molecules has also received attention. Measurements by the more direct methods tend to give values in the range 7×10^6 to 15×10^6 for *Hemophilus* (64, 151). Smaller estimates have been obtained by x-ray inactivation: 7×10^5 for the TP for streptomycin resistance in *Pneumococcus* (47). Radiation experiments are also of interest in showing heterogeneity of transducing DNA; the former estimate refers to the smaller type of particle. Different markers for pneumococcal transformation were found to be inactivated by radiation at the same rate, sug-

gesting similar size (120); they all showed the same type of inactivation curve, which could be explained by the existence of transforming particles of two molecular weights, one smaller than 10^6, and the other larger than 2×10^6. While it would require data of greater precision than those now available to establish that only two sizes of particles are present, it is of considerable interest that the particles capable of joint transductions, and therefore possibly corresponding to genetically larger fragments, seem to show the radiosensitivity pattern of the larger type of particles (120).

Inactivation of TPs with other agents.—Ultraviolet inactivation gives a multi-hit curve (180); photoreactivation can occur, provided the photoreactivating system is supplied with an extract from *E. coli* B cells (65). Most strong mutagens inactivate TPs even in minute concentrations, supporting the idea that DNA is the site of action of mutagens (180). The kinetics of inactivation by heat, and the behaviour on storage after treatment, suggest that labilization precedes inactivation. It was tentatively postulated (180) that the direct effect of these treatments on DNA is the breakage of a few hydrogen bonds, which is sufficient to make the DNA molecule labile to other external agents. This explains delay in mutation after application of mutagens. A similar conclusion was reached in a study of the effect of water content of cells on killing, mutation, and DNA gelation in *Serratia* (88, 89).

Physiology of the process: cell competence.—The competence of cells to take up transducing DNA is limited both genetically, and by physiological and environmental conditions. Transformability differs between strains belonging to what is considered to be one species of the *Hemophilus* group; within the strains of one species it is mostly a property of the recipient. Poor recipients are poorly transformed even by their own DNA (106). In highly transformable strains it is known that all cells are potentially capable of undergoing transformations; but only a few cells are transformable at any given time. The highest recorded rate of competent cells is 17 per cent (78); but the usual fractions are between 1 per cent and 1 per thousand.

The development of competence has a peculiar kinetics. Peaks of competence are observed during the growth of a culture, and in some conditions a periodicity is shown (167). Transfer to fresh medium blocks competence which reappears only after some time; the time lag is a function of the degree of dilution. In agreement with earlier observations by Hotchkiss, the period of competence of a cell is brief (15 min. or less). During this period the cell is permeable to large molecules, such as DNA, and even deoxyribonuclease. The disappearance of deoxyribonuclease sensitivity, in fact, coincides with that of DNA transducibility.

Competence in *Hemophilus* populations appears to reach a peak shortly before attainment of the stationary phase (147). This fact may hamper the discovery of transformations, if the phenotypic expression of the transformd cells should require further growth.

Some of these facts are not easily reconciled with the previously expressed

view [see (46) for a fuller discussion] that competence varies cyclically with cell division, there being a phase of the division cycle highly competent for transduction.

Physiology of the process: DNA uptake.—All kinetic experiments (46, 167) agree with the earlier ones (140) in demonstrating that one molecule of specific TP is sufficient to bring about transformation of one cell. Only a fraction of the DNA employed is irreversibly adsorbed by the cell; the rest can be washed off by saline or attacked by deoxyribonuclease after adsorption [*Hemophilus* (64)]. The total number of DNA molecules taken up for each cell transformed has been estimated to be about 100. This may be related to the fact that there should be about 100 molecules of DNA per donor cell, one only of which corresponds to the specific marker selected in the transduction experiment (64).

At concentrations of DNA above 10 molecules per recipient cell, the number of transformations is less than expected. Competition or interference between molecules of DNA is suggested; it could occur if there were an upper limit to the number of DNA molecules that can be taken up by a cell (64). Previous evidence of inhibition of transformation by foreign DNA may be connected with this phenomenon.

Within 10 min. after adsorption, transforming DNA can still be recovered by lysing the cell with lysozyme; it is biologically active and deoxyribonuclease-sensitive.

Phenotypic expression and segregation.—Phenotypic expression may take place as early as 30 min. after DNA uptake [for drug resistance in *Pneumococcus* (78)]. In this species, streptomycin resistance seems to be dominant, rather than recessive to sensitivity as in *E. coli.* Dominance is suggested by the early appearance of the resistant phenotype, and also by the recovery of sensitive segregants from cells transformed to resistance, which were actually tested for resistance at an earlier stage (79). Segregation was shown to continue for a few generations; thus, genetic incorporation need not take place immediately after transduction but, as in some cases of phage transduction, a "heterogenotic" stage, (heterozygous for the fragment), may persist for some time. With the transduction of dominant markers, phenotypic expression may therefore precede segregation; linkage estimates may be affected by it.

Genetic linkage studies.—Streptomycin resistance in *Pneumococcus* is linked not only to mannitol fermentation, as previously reported, but also to sulfanilamide resistance (120). A weak linkage between streptomycin resistance and encapsulation factors has also been announced (142).

The assessment of linkage is of questionable reliability. It was shown to be seriously affected by the agglutination of recipient cells (142). Furthermore, since only a small fraction of the cells are competent, the frequency of joint transductions of unlinked factors should be higher than the calculated product of single transductions and not equal, as is usually assumed. If it is not higher, disturbances (e.g., interference) must be at work. Physical

aggregation of particles in DNA preparations may simulate linkage, even in comparisons between joint transduction with DNA prepared from the doubly marked strain, and with a mixture of DNAs from the two singly marked strains. Only close linkages, such as those of mannitol and streptomycin, or of capsular factors between themselves [as shown, e.g., from "allogenic" transformations (46)] are easily acceptable until more detailed data and proofs are available.

Heterospecific transformations in Hemophilus.—A difference in the transforming effect of homologous and heterologous DNA has been ascertained in transductions between *Hemophilus influenzae, H. parainfluenzae, and H. suis* (106, 147). The lower transforming activity of heterologous DNA can, however, be restored to the normal homologous level after its incorporation into the homologous genome; i.e., DNA from streptomycin-resistant *H. influenzae* is equally effective in determining transformation to streptomycin-resistance of *H. influenzae*, independently of whether it is obtained from a resistant mutant of *H. influenzae*, or from a strain of *H. influenzae* transformed to resistance with a DNA from *H. parainfluenzae* (and vice versa). The titer of heterologous transducing activity is, instead, influenced by the ancestral origin of DNA. In the same example as above, DNA from a resistant mutant of *H. influenzae* is less effective than DNA from an *H. influenzae* strain originally transformed to resistance with the DNA from an *H. parainfluenzae* strain, in determining resistance transformation of *H. parainfluenzae* (106).

As concluded by the authors, it is likely that the linkage relationships of TPs, in the original chromosomes are partially kept in the extracted DNA, and are responsible for this behaviour.

Other problems and species.—The transforming activity of DNA for mannitol fermentation (which is due to an adaptive enzyme) has been shown to be independent of whether the donor cells were in the adapted or unadapted state at the time of DNA extraction (121). Two R mutants of *Pneumococcus* type VIII are nonallelic, as each can transduce the other to VIII S, an extreme case of "allogenic" transformation (128). Various results were obtained when these strains were transduced with DNA from other pneumococcal types. Doubly encapsulated pneumococcal types (III+I and III+V) have been synthesized by transformation (3).

Mutations to streptomycin resistance conferring different degrees of resistance were stated to be allelic (148).

DNA transduction has been reported in two new species: *Xanthomonas phaseoli* (30) and *Agrobacter tumefaciens* (92).

General reviews: (46).

PHAGE MEDIATED TRANSDUCTION

In the transformations so far discussed, DNA is transferred from the donor to the recipient in a naked state. In phage mediated transduction the carrier of genetic information is presumably also DNA, but the DNA is

inaccessible to the action of deoxyribonuclease and to direct chemical investigation, because it is carried within a phage coat. Thus, phage can act as a carrier of chromosome fragments from the cell which it has lysed in its last growth cycle, to the cell which it will infect next, provided the new infection does not terminate with the host's death. It is useful to distinguish two types of phage-mediated transduction.

The first is a generalised, or nonlocalised type. Any marker from a bacterium can be transduced in this system, which has been studied in the genera *Salmonella*, *Escherichia* and *Shigella*. The transducing property is not uncommon among temperate phages; thus, out of 115 freshly isolated *Salmonella* strains, of which 62 per cent were lysogenic for a phage active on *Salmonella typhimurium* TM_2, 14 carried phage with transducing ability (4). It was somewhat more difficult to isolate transducing phages for *E. coli* K-12 (83). Transducing ability is not necessarily a property only of temperate phage. At least in *Salmonella*, virulent phage can transduce, provided the recipient cell is protected from lysis either by being lysogenic for the homologous, temperate phage, or by being simultaneously infected with a higher multiplicity of lysogenizing, nontransducing phage (182).

The second is a limited, or localised type. Only one example of this type is known: lambda phage transduction in *E. coli* K-12. This phage can carry only a limited group of markers, all having a common physiological function (galactose fermentation), closely linked to one another and to the lambda prophage itself. Here, the spatial relations between location of the phage on the chromosome, and the segment transduced are clear. The phenomenon might be more common than is actually known; the chance of discovering such a localised transduction, i.e., of detecting the right markers, will in general be a small one.

Salmonella: generalities on transduction.—Transduction may be envisaged as the injection into the recipient, by the transducing phage, of phage DNA together with bacterial DNA resulting from the lysis of the donor cell. When these two types of DNA have entered the recipient, they seem to undergo somewhat independent fates, each having certain probabilities of giving rise to one of a series of possible events. Phage DNA may lyse, or lysogenise, or fail to perform either of these processes; bacterial DNA may or may not be incorporated. Moreover, bacterial DNA found in phage is not transferred genetically by the phage itself; apparently all the bacterial DNA in the phage is the outcome of the last growth cycle (182).

Independence between transducing and lysogenising ability is further shown by their different response to ultraviolet and other types of radiation. Transducing ability of phage is increased by ultraviolet irradiation, while its lysogenising capacity is inactivated even faster than its lytic activity. The response to decay of incorporated P^{32} is a different one. Lytic and lysogenising abilities are destroyed at the same rate; transducing ability at a rate about ten times lower (59).

Moreover Zinder (182) has followed the kinetics of incorporation of

bacterial markers into phage during growth of the latter. All markers tend to reach a similar plateau value of the frequency of transducing particles per phage particle (3 of the former per 10^6 of the latter). The rate at which this ratio increases during phage growth is similar for various markers, and similar to the growth rate of phage itself; but the starting point in the rise of the ratio of transducing particles to phage particles is different for different markers. Some are already at or near the plateau at the shortest time investigated; others, the slowest ones, have not reached it at the latest time investigated. The behaviour of different markers could be explained by assuming that the breakdown of the bacterial chromosomes during phage growth takes place in a sequential manner, and the availability of this material for inclusion into maturing phage particles reflects such a sequential breakdown.

Considering the burst size of this phage, the yield for any given marker, at the endpoint of phage growth, has been calculated to be about one transducing particle for every three to five hundred bacteria.

Genetic linkage in Salmonella.—Joint transduction has been observed consistently in a systematic survey of auxotrophic mutations by Demerec and co-workers, and in view of the importance of such results for the study of gene action, they will be discussed in a special section of the chapter dedicated to physiological genetics, along with other transduction work of physiological significance.

Abortive transduction.—When DNA has entered the recipient cell via phage, it is occasionally able to exert its specific phenotypic action without being able to multiply. Unstably transformed bacteria will result: the gene fragment or "endogenote", unable to duplicate, will be passed on to only one of the daughter cells at every division. Clones originating in this way contain only one cell which possesses the gene in question, a situation called "unilinear inheritance." Abortive transduction for a motility-conferring gene (105, 161) is the explanation offered for the origin of "trails," tracks of colonies of nonmotile or scarcely motile cells in experiments of transduction for motility in semisolid agar. In the same conditions, fully successful transductions give rise to "swarms" of fully motile cells. On closer analysis, the picture is found to be of some complexity (137, 138, 139, 162, 163), but the observed results can be fitted into the following scheme (162). The transduced, nonduplicating gene synthesises motility-conferring particles in the cells which carry it. These particles are probably flagella, or the basal granules producing flagella; they cannot duplicate and are distributed at random to the progeny. Thus, at the division of a gene-containing cell, one daughter will receive the gene plus a number of motility conferring particles. This cell will be phenotypically motile, and capable of reproducing the process of unilinear inheritance. The other daughter cell will receive only the motility-conferring particles; it will also be motile, but a dilution of the particles will occur in its progeny, until some cells will have one particle, and others none. It has been estimated that one "gene" can synthesise about 15 particles in one generation; one particle appears to be sufficient to confer motility in

broth, but several are necessary for a cell to be able to travel in agar. The "trail" is formed by the abortively transduced, motile progenitors which deposit their nonmotile or insufficiently motile sisters along their path. These will form localised, nonswarming colonies.

Abortive transduction has been described also for a biochemical marker, in experiments with an adenine+thiamine auxotroph (134). In these abortive transductions (which are more frequent than successful ones), very minute colonies, each containing only one prototrophic cell, are formed on minimal agar. Only the prototrophic cell reproduces normally in minimal medium, passing on the nonduplicating gene to one daughter cell. The other daughter cell carries, however, some enzyme and can perform a limited number of divisions (especially in adenine pantothenate medium), but will eventually return to full auxotrophy.

Generalised transduction in E. coli.—Bertani's phage P1, capable of lysogenising strains K-12, B and C of *E. coli*, and various *Shigella* strains, was found (107) to be able to transduce practically any of the usual markers, including lambda prophage, with a frequency of approximately $10^{-6}-10^{-8}$ transducing particles per phage particle.

K-12 markers shown by genetic analysis to be closely linked can be transduced jointly, and the order of genes from chromosome maps obtained in sexual analysis is confirmed by transduction. The size of fragments transduced is unknown, here as elsewhere; but from the data it might be estimated to be not too far from 1/100 of a bacterial nucleus (a rough estimate, based on the frequency with which markers are found to be linked in transduction). Lysogenisation is to some extent, independent of transduction; it may occur at a higher frequency, however, when selection is carried out for a certain group of markers (fermentation markers in the Streptomycin region). This is the only hint of a localisation of the prophage itself. In contrast to the situation in *Salmonella*, clones from transduced cells or K-12 show a long lasting segregation of transduced markers, suggesting a persistence of the heterogenotic stage and, frequently, late incorporation.

A phage related to P1 was independently found to transduce ordinary markers in K-12, as well as three different prophages [one of which is lambda as mentioned previously (83)]. It was the only phage transducing out of 23 tested. Transfers of both lysogeny and nonlysogeny have been obtained. Lambda phage mutant markers have been transduced and tentative linkage between one end of the lambda phage map and a bacterial marker has been proposed. The other two transducible prophages are both linked to a Gal marker, to which also lambda is linked.

Localised transduction: Lambda phage in E. coli K-12.—This unique instance of localised transduction, discovered a few years ago, has been further investigated (124, 125, 126). The markers known to be transducible affect galactose fermentation; one Gal marker (Gal_6) is, however, not transduced. All transducible loci are usually found to be nonallelic, both by sexual and by transduction analysis. Transduction affords a simple test of physiological allelism, since "heterogenotes," i.e., bacteria carrying a transduced

fragment, can multiply as such for a fair number of generations. Some *trans* double heterozygotes for two Gal markers $(+-/-+)$ can ferment galactose; others (Gal_1, Gal_4) cannot. A physiological analysis is stated to be in progress; preliminary reports suggest that the transducible fraction of Gal markers is concerned with the manufacture of at least two different enzymes.

Any one of three events may occur when heterogenotes divide. The first is reduction to the haploid state, with or without crossing over. Stable clones are thus produced. Reduction takes place once every 10^3 cell divisions. Secondly, heterogenotes may be propagated unaltered by a process akin to mitosis; thirdly, they may undergo "automixis" (internal recombination), to form a new heterogenote, possibly by a process similar to mitotic crossing over. Automixis is clearly observed in Gal_1 Gal_4 dihybrids; from the *trans* $(+-/-+)$ heterogenote, which is unable to ferment galactose, the galactose-fermenting *cis* $(++/--)$ heterogenote can originate no matter whether the $++$ moiety is in the fragment or in the main chromosome. Automixis occurs in a way that implies crossing-over at the four-strand stage when chromatids have duplicated.

In addition to their capacity to segregate, heterogenotes possess another peculiarity. Lambda induced by ultraviolet in heterogenotes has a high frequency of transduction (HFT), perhaps 10^5 times higher than that of normal lambda particles (about 10^{-1}). HFT lambda transduces preferentially the "exogenote" (the fragment). The probability of genetic incorporation of the fragment by a recipient is unknown but if it is of the order of 1 in 10, one could conclude that all HFT lambda particles carry a transducible fragment. Estimation of DNA content of normal and HFT particles would be of interest. The distribution of fragment sizes is not known, but might be relatively homogeneous.

Sexual Transfer

A mechanism of genetic transfer, requiring cell-to-cell contact, was first described by Lederberg and Tatum in 1946, and is often referred to as "Bacterial Recombination." Phenomena of this kind are no longer restricted to *E. coli*, although every species investigated successfully has peculiarities of its own. Whether or not these processes should really be considered sexual ones, depends on one's definition of sexuality. However, collected under this heading are all mechanisms which seem to require cell-to-cell contact and, therefore, presumably, conjugation. At least, the operational basis of the classification is clear.

Conjugation, preliminary to genetic transfer, has now been clearly observed and documented by Lederberg (101). Further cytological work has been announced (1, 176). In the admixture of fertile strains pairs of cells form; the use of strains morphologically distinguishable (by motility and cell shape) shows that the pairs thus seen are legitimate. After about an hour partners separate. Both exconjugants are usually viable, but only one of the two cell types (the "female," F−) has recombinants in its progeny; the other parental cell (the "male," Hfr) is unaltered. Thus, in *E. coli* conjugation

(transfer from one parent to the other) takes place; but a double, reciprocal fertilization, as, e.g., in *Paramecium*, is not observed. Unidirectional transfer is in agreement with what had been earlier postulated on the basis of indirect evidence (Hayes), but no specific vector seems to be involved; at least, no extracellular stage of the male gamete has been detected. A whole cell normally functions as a male gamete (donor) and a whole cell as a female gamete (acceptor). However, the "donor" is not used up in the process as it is in all cases of transduction so far studied. Presumably the survival of the donor reflects the multinucleate condition of the cells, which permits the loss of a complete nucleus without a loss of viability.

We shall apply the terms "male" or "donor" strains to strains which can act potentially as such (F+, Hfr), although it was known from earlier work that, under certain conditions, they can act also as females and are therefore usually self-fertile or homothallic. In contrast, "female" or "acceptor" strains (F−) can only act as such; they are self-sterile.

Initially, cell-to-cell contact is probably the result of chance collisions, but the maintenance of the contact by a connection must reflect specific properties or activities of the cells participating in conjugation. Differences between the cell surfaces of male and female strains have been demonstrated (117, 118, 119). Suspensions of female cells are optimally precipitated at a lower pH than are male cells, independently of related effects due to smooth-rough dissociational types. Such surface differences are probably reflected in the changes of turbidity which cultures of male and female strains undergo during growth; and also in the stability of suspensions of cells, which is higher for the female type, male strains often agglutinating spontaneously. Female strains show also a greater affinity for basic dyes than do male strains (117, 119). Moreover, an antigenic difference in terms of K antigens has been reported (118).

These cell-surface differences are probably relevant to the phenomenon of conjugation, since agglutination occurs on admixture of male and female cells (24). Conditions must be chosen such that spontaneous agglutination of the male cells does not obscure the phenomenon. "Sexual" agglutination has not been obtained so far with killed cells, which makes an analysis of the cell surface components involved a difficult one, and suggests that an interaction between cells of the two types develops upon contact. This is further substantiated by the fact that energy is required at this initial stage (51). Recombination is also possible between penicillin protoplasts (103).

Transfer of genetic material.—The actual transfer of genetic material across the bridge connecting male and female takes place in the following way according to Wollman & Jacob (174). Rather than the injection of a male nucleus into the female, the transfer of a male chromosome (or a segment of it) occurs slowly and gradually, one end always first; as if, to use a rough comparison, a recording wire carrying the genetic information were unwound from a reel in the male and wound up again on a reel in the female. The transfer requires practically the whole time of conjugation, almost an hour. This hypothesis has evolved on the basis of experiments in which con-

jugating cells were, at intervals after mixing, agitated in a Waring blendor. The treatment left the cells undamaged, as far as their viability was concerned, but was assumed to break conjugating pairs, severing the bridge across the two partners and interrupting the exchange. The "interruption of transfer" by mechanical agitation at an early time during the process caused the absence or decrease of recombinants, carrying, e.g., the genes T, L, while treatment at later times did not prevent their incorporation but was effective on markers further along the chromosome. A chromosome map made by "time analysis" gives a gene order superimposable on that formerly obtained by genetic analysis.

The results of timed shaking of conjugal pairs can be interpreted on the theory of slow and gradual transfer of a chromosome segment, at least for the markers investigated. Another explanation of these findings has been offered: namely, injection of a whole nucleus in an orderless fashion and interference by mechanical shaking with the process of pairing, or duplication process of the chromosome in the zygote, or both, assuming these to have a constant order (98). Further work has, however, supported the original hypothesis of Wollman & Jacob (174). The timed interruption of transfer can be accomplished irreversibly by timed killing of the male parent with T_6 phage, to which the female is resistant (74, 177); and reversibly, by exposure to agents blocking energy utilization by the male, which is the only active partner of the transfer (51, 177). Even mechanical shaking, much milder than exposure to the Waring blendor, may be sufficient to block the transfer (74). Probably the best evidence in favour of the gradual, ordered transfer theory is the observation that exactly the same relative order of genetic loci on the male chromosome can be established by decay of P^{32} atoms incorporated into the chromosomal transferred DNA as had been previously established by mechanical interruption (177).

Energy required in the transfer (by the male) has been found to depend mainly on the Krebs cycle. Inhibitors of carbohydrate oxidation therefore prevent the process which is not sensitive to antagonists of protein and nucleic acid synthesis (50, 51).

Partial or complete transfer?—It was found earlier that the genetic contribution of the male and the female parent to the progeny is unequal, the male always contributing less than the female. It remained to be decided whether this was caused by incomplete transfer (prezygotic elimination), selective destruction, or nonincorporation of male genetic material injected into the female (postzygotic elimination) (98, 177). The evidence from interrupted transfer, even accepting the hypothesis of chromosomal segment transfer, is not directly relevant to the events taking place in uninterrupted conjugation.

Agreement has been reached, however, on the existence of "critical regions,' eliminated with high selectivity (25, 26, 27, 98, 175, 177) presumably because they suffer breaks in the male chromosomes. Thus, fragmentation (or inactivation by other means), absence of male fragments in the zygotes, and poor synapsis ("pairing") of fragments, may all contribute to the

asymmetry of the contributions of the two mating types to the progeny. Results from haploid segregants cannot clearly distinguish between the two latter possibilities (25, 98). Effects of chelating agents on transfer have been reported (44).

It may also be mentioned, although the information is preliminary, that the asymmetry in the contribution of the males and females need not be general. A fertility agent (F_2) from a strain other than K-12 seems to confer a behaviour in K-12 reciprocal crosses which is practically free from asymmetry, or at least not comparable to that observed with the original F agent of K-12 (10).

Zygotic induction.—In crosses between some *Hfr* lysogenic×nonlysogenic females, prophage is induced and develops in a fraction of the zygotes which are therefore lost (86). This may cause a certain selection of recombinants and is, according to the authors, further proof of the theory of partial transfer. Only prophages which are inducible by ultraviolet are induced in the zygotes.

Variety of male types.—Two male types have been described, F+ and Hfr. The former is capable of donating the F+ capacity to F− strains with a high frequency, ("F+ infection"), but recombines with them at a low frequency (10^{-5}). Hfr cannot convert F− strains to the F+ or Hfr condition, but recombines with them with a higher frequency than does F+; the frequency varies somewhat with the markers and the Hfr, F− strains employed. Hfr is transmitted only rarely to recombinants from Hfr ×F− crosses. Linkage has been shown between Hfr_1 and Gal_2 and between Hfr_2 and B_1 (25, 26, 27); both these Hfrs and the markers linked with them are preferentially eliminated in the crosses.

Jacob & Wollman (85, 175) have advanced the theory that the difference between Hfr and F+ strains is due to a different location of a "gene" (let us call it the F gene), which has the property of determining male capacity and also breaks the chromosome in its neighborhood at transfer. In F+ strains, F is located near that chromosome end which enters first, so that only F and the short segment ahead of it can be transferred. In Hfr strains the F locus is transposed by mutation, to some other part of the genome and a longer segment of chromosome is now transmissible at conjugation. The main support for the theory comes from the following evidence. In "time analysis" F+ is transferred from F+ to F− in the first few minutes, preceded by only one marker, colicin E production (which is transduced along with F+ in "F+ infection"). A set of parallel F+ cultures gives a positive fluctuation test for number and type of recombinants. By replica plating it is possible to select, in sites corresponding to those of fertile cells in F+ strains, Hfr clones. These Hfr clones show differences among themselves in the range of markers transferred with high frequency. If the theory, which is undoubtedly attractive, is right, several facts will need auxiliary explanation. Some of them have been already commented upon in the paper quoted (177).

Segregation.—In the great majority of zygotes, segregation occurs fairly

early, although there are no data yet which show that a regular meiotic mechanism is at work. However, it has been shown (74) that a recessive gene like T_1 phage resistance, entering the zygote from the male, begins to express itself soon after the beginning of division of zygotes (120 min. after conjugation), and that its phenotypic expression is practically complete after 300 min. (four generations). Nuclear segregation can thus explain most of the phenotypic delay. Azide resistance instead manifests itself soon after the genetic determinant has entered the zygote, and expression is complete when the division of zygotes begins, suggesting dominance of the resistance allele.

Various other results.—Stable small colony variants, obtained by copper treatment in K-12, segregated in crosses with wild type; the existence of various loci determining the change was indicated (29). Resistance to colicin E was linked with the B_1 marker (87). The inheritance of colicin production (see also above) follows unusual patterns, suggesting transduction mechanisms (55). An unexpected identity of the loci controlling maltose fermentation and resistance to phage lambda (Lp_2, not allelic with the prophage locus) was found (97). Cultivation in the presence of nickel or cobalt has altered the mating type of F+ cells to F−; the change is not correlated with resistance (75).

Outcrossing of E. coli K-12.—Crossability of *E. coli* B and K-12 had already been reported (181) and has been independently observed (33). F+ "infection" of *E. coli* B by K-12 F+ was reported after prolonged mixture (33) but (rare) transfer by crossing is known to be possible (22). Radiation resistance and sensitivity segregated in crosses of B/r and K-12, both parents having a similar degree of radiation resistance (19). Multiple phage resistances segregated in crosses as unit mutations; B/3, 4 is not allelic to B/3, 4, 7 (34). Effects of crossing conditions (35) and delayed segregation complicate the picture (19).

E. coli C, a strain closely related to NCTC 123, previously known to cross with K-12 possesses a cell and nuclear morphology distinct from those of K-12. Both external cell form and nuclear morphology segregated in crosses (111).

Pseudomonas aeruginosa. Crosses were set up between four strains marked with a total of 18 auxotrophic mutations (76). The four strains were interfertile at a low rate in certain combinations of markers. No evidence was found to indicate genetic transfer in conditions excluding cell-to-cell contact. Recombination for unselected markers has been obtained. Self-fertility of strain L was shown in a later report (83), and self-sterility of strain 1 was confirmed. No sign of F+-like agents were detected. Slight evidence of fertility in this species was independently reported (114).

Serratia marcescens.—A system of genetic exchange requiring cell-to-cell contact has been described in which transfer of more than one marker at a time is not detectable (8). Crossing experiments rely therefore on selection with single auxotrophy markers and no recombination of unselected markers has been observed. The treatment with ultraviolet of one or the other of the strains crossed markedly enhanced prototroph formation. Results have

been interpreted as being caused by the transfer of chromosome fragments in a system requiring cell-to-cell contact. Every strain tested is capable of behaving both as a donor and as a recipient. Ultraviolet irradiation enhances the donor activity of the irradiated parent.

Streptomycetes.—As has been previously mentioned, the formation of recombinants after a heterokaryotic stage has been safely established in *S. coelicolor*. The details of the process are not yet clear, but the simultaneous transfer of a number of markers has been ascertained (150). These results have been confirmed and extended (165).

Lysogenic Conversion

Conversion to lysogenicity may be considered to be a particular type of genetic exchange. Cellular reactivation of lysogenic activity in phage subjected to ultraviolet irradiation is supported by data on phage P22 in *Salmonella* (59). It is considered to result from the existence of a section of phage chromosome homologous with a section of the bacterial chromosone. From radiation data, it has been suggested that the homologous region constitutes the largest part of the genetic material of P22.

A study of the fate of DNA of temperate phage P1 during lysogenisation of *Shigella* (63) using P^{32} as a marker, has shown that practically all phage DNA enters the lysogenic progeny where it is retained for at least 8 to 11 generations (in the preprophage state?). An analysis by P^{32} decay of the lysogenic activity of lambda (160) has shown that very little, if any, of the injected phage DNA is actually incorporated into prophage.

Temperate phage entering a sensitive bacterium behaves similarly in its first stages, whether following the lytic or the lysogenic route (160). Preprophage (unintegrated lysogenic phage) can undergo genetic recombination before reduction to the prophage state (109).

Prophage sites in the bacterial chromosome.—The number of sites on the bacterial chromosome which a prophage can enter or to which it can attach itself, may be, in some strains, more than one. Even triply lysogenic strains for phage P2 were obtained in *Shigella*. Doubly lysogenic strains crossed to sensitives yield four types: nonlysogenics and doubly lysogenics (parentals), in addition to singly lysogenics (recombinants). Of the possible attachment sites, one is preferentially occupied, and when a doubly lysogenic strain is superinfected with new phage, prophage substitution may occur at the preferential site. Prophage located at the preferential site has also a greater chance of multiplying. Interaction between prophages present in different sites of the same cell does take place as shown by recombination between them (11). In P1, only one prophage site is indicated by P^{32} work (63).

Two phages from *Shigella*, lysogenising K-12, were mapped in sexual crosses (49); one of them has a locus close to lambda. The other has a different location, not far from C-St markers. Recombination has, however, been observed between independent lysogenics for this prophage, and there again more than one prophage locus may be indicated.

Phage D (from *E. coli* W) lysogenising K-12, has also a locus close to

lambda (152). It may be noted that several phages have a preference for this chromosome region which is related to the "critical region" detected by recombination analysis.

Changes correlated with lysogenic conversion.—In addition to the specific effects of lysogenisation, other changes may accompany it; these include alteration of surface properties and, most interesting of all, toxigenicity (in *Corynebacterium diphtheriae*). Recent advances in this problem will be discussed below (69, 70).

Further work on the transfer of toxigenicity in *C. diphtheriae* has confirmed the observation that the converting ability of phage is inseparable from phage itself, and is independent of phage propagation on a toxigenic host provided care is taken to avoid phage mutation. Lysogenisation by phage β of any of the strains sensitive to it is always accompanied by toxigenic conversion. The reverse process, loss of lysogenicity, has always been accompanied by loss of toxigenicity; sensitives obtained can be reconverted by phage to toxin production. The discovery of the new phage, γ, closely related to β, capable of lysogenising the *C. diphtheriae* strain C4, but incapable of converting it to toxigenesis, has been reported. Recombinants between β and γ prophages have been described. One of them, β', is incapable of toxigenic conversion, but retains the host range of β, another, γ', is capable of toxigenic conversion but retains the host range of γ. This may be a clear cut example of phage genes having a definite effect on bacterial metabolism; the boundary between phage and bacterial genes is becoming thinner and thinner. The toxigenic gene is "dominant": bacteria doubly lysogenic for β and γ are toxin producers.

Defective lambda mutants.—Defective lambda prophage can produce only $10^{-5}-10^{-7}$ phage particles per induced bacterium (2, 86). The defect can be transmitted to mature, extracellular lambda (2). A locus responsible for one such defect has been mapped on the lambda map, by superinfecting ultraviolet-induced defectives with lambda carrying suitable mutants (86). The defect seems to lie in the maturation of the phage particles. Phage genetic material multiplies normally after ultraviolet induction; empty phage coats resembling "doughnuts" are found in lysates.

A hint has been given (6), that phage from virulent *Agrobacter tumefaciens* may cause crown gall tumours in carrot. If confirmed, this phenomenon might prove of the greatest interest.

GENETIC CHANGES IN INDIVIDUALS AND POPULATIONS

Spontaneous Mutation

Mutation rates and metabolic conditions.—In complex media, mutation rates are higher than in simpler ones, and insensitive to mutagens and antimutagens of the purine type (53). At high growth rates, moreover, they show dependence on generation times, in contrast with independence previously observed (Novick and Szilard) at low growth rates in the chemostat (42, 53). Such effects may be the consequence of control on mutation rates by metabolic conditions.

Further support for this idea comes from an analysis of spontaneous mutations in nondividing bacteria (144). During the stationary phase, mutants $h- \rightarrow h+$ accumulate, being produced at a constant rate, about forty times lower than in rapidly dividing bacteria. This seems to be the result of true mutation in the resting stage and not of phenotypic delay in mutants which had arisen earlier, or slow cell turnover in stationary conditions of the culture. A similar effect of metabolic state may explain preincubation effects on mutants arising in *Streptomyces* spores stored in the cold (169).

Mutator genes.—Two examples of high mutability have been described, both in *E. coli.* The recently detected "Harvard strain" has been shown to give rise to auxotrophic mutants at a remarkably high rate (estimated at 1.5×10^{-3}). Even double auxotrophs arise spontaneously with a frequency similar to that with which single auxotrophs would be found in normal strains. Ultraviolet causes no further increase in this rate (62).

A strain derived from K-12, discovered by Treffers *et al.*, which also shows high mutability (the "Yale strain") has been subjected to genetic analysis (19, 153). Mutability segregates as if due to a single locus. The Mut gene was mapped in the sequence: V_1-Mut-L. Streptomycin-resistant mutants induced by Mut are indistinguishable from normal ones. Mutability of the Yale strain is decreased under anaerobic conditions (19).

Drug-resistance.—A number of reviews and general articles have appeared on this subject (20, 21, 37, 131, 170, 171, 173). Experimental contributions have employed mainly indirect selection methods, and will be discussed in the next section.

An investigation was made of the variability of first-step, penicillin-resistant mutants (122). Various degrees of resistance are found, generally accompanied by depressed growth rates compared to that of wild type. In penicillin, growth was even slower. Subinhibitory concentrations of the drug in agar were ineffective in bringing about resistance, while in broth they were rapidly effective in selecting resistant mutants. Similar observations on proflavine resistance have received a different interpretation in a paper (32) which renews and extends criticisms of the tests of genetic adaptation. A more powerful means of demonstrating genetic adaptation has meanwhile been developed (28). Since it has fully confirmed previous conclusions, and will be outlined later, a detailed answer to these criticisms is not necessary here.

Indirect selection.—The implications of the replica plating technique have been misunderstood (94, 112, 149, 172), but these misunderstandings have been already clarified (61, 154). It should be stressed that replication is a sampling procedure, and that only a sample of a colony is transferred while another sample remains on the master plate. The population sampled is heterogeneous, as any clone, especially if it has reached a large size, is bound to be. (Actually, some pedigrees of single cells (80, 81) tend to show an extreme heterogeneity but this variation is observed under conditions which make its actual estimation difficult, and possibly increase it artificially). Heterogeneity in the population sampled is, in any case, an essential pre-

requisite for the success of indirect selection. The total extent of the heterogeneity of a population is difficult to assess, but it is established that the variety of mutant types of any particular kind is large. The only conclusion to be drawn from certain experiments reported (112, 149, 172), is that different modes of selection, differences of selection intensity and variations in environmental conditions under which selection occurs, affect the probability of detection of the various mutant types.

Mutation to penicillinase production in *B. cereus* has been investigated by indirect selection with replica plating. On three separate occasions, mutants capable of producing penicillinase constitutively were indirectly selected (154).

A new method which makes possible indirect selection of mutants in liquid media has been described (28). It is more amenable to quantitative analysis than replica plating; by its use, one can ascertain whether all mutants detected by direct selection are recoverable also indirectly, i.e., are spontaneous mutants. It links the principle of sib-selection, of which use is made in replica plating, with a method of concentrating desired mutant types, which is described below.

From a culture containing x resistant mutants, as determined by counts with drug plates on samples from it, a sample expected to contain one (or a few) resistant mutants, is divided into n fractions, each of which is inoculated into a fresh tube. Only one (or a few) tubes will then contain a resistant mutant. Such tubes can be identified by testing a sample on drug plates from all n tubes after incubation. In such a tube an enrichment of mutants is expected (i.e., an increase in the relative frequency of mutant cells in the population) which is equal to n, the number of tubes into which the inoculum with one mutant was subdivided. A few such enrichment cycles may yield the mutant in pure culture. A chloramphenicol-resistant mutant and a streptomycin-resistant mutant of *E. coli* were successfully selected indirectly by this procedure. Actually, the process is somewhat slowed down by the slower growth rate of resistant mutants, but observed enrichment was nevertheless in agreement with expectation.

Techniques.—Papillation of colonies has, since the very beginning of bacterial genetics, been considered an indication of overgrowth by mutants. Counting papillae corresponds to counting mutations rather than mutants, an advantage easily appreciated by anyone directly acquainted with the vagaries of the numbers of mutants in the Luria and Delbrueck, Lea and Coulson distribution. The method of counting papillae has been adapted to the study of mutations to prototrophy, using suboptimally supplemented minimal medium (146). The penicillin technique can be employed for selecting fermentation mutants (48).

Mutagens

Specificity of mutagens. Ultraviolet and x-rays, nitrogen mustard, diepoxybutane, and triethyleniminotriazine were tested for mutagenicity on a panel of 24 different mutations in *E. coli* (60). Mutagen specificity has been

confirmed, but some correlation exists between most of these mutagens. An effect of genetic background on mutagen sensitivity was clear cut. $MnCl_2$ has an action of its own uncorrelated with that of the above agents.

An analysis (71) of sensitivity to mutagens in 8 Gal- and 15 serineless mutants in *Salmonella* has revealed a similar degree of specificity; $MnCl_2$ was, however, almost ineffective. Spontaneous and induced mutabilities were correlated. Mutagen stability and mutagen specificity are individual properties of the single alleles, no correlation being found between "nonidentical" alleles.

Phenotypic delay.—Phenotypic delay in reversion to prototrophy of lysineless *Salmonella* has been studied in (90). It was shown by Newcombe's respreading technique that mutants do not begin multiplying until $6\frac{1}{2}$ hr. after ultraviolet irradiation, while parental cells have a lag of only 3 hr. Two hypotheses were entertained: (*a*) mutants remain dormant during the interval; (*b*) they multiply regularly after the third hour, but the mutant chromosome acts as such only several generations later, meanwhile being transmitted unilinearly. A test was set up with penicillin and showed no enrichment in the ratio of mutants, supporting the second hypothesis.

On the basis of analogies between ultraviolet and x-irradiation, it was suggested that x-ray-induced mutations also may be delayed, and possibly the consequence of a multistep process (130, 132). A discussion of phenotypic delay was given (145).

Population Genetics

Orthoselection is the name which was given some time ago by Ryan to the prevention of the accumulation of mutants in a population by the occurrence of other mutations, having a selective advantage over the parental type. In crosses between strains, of which one or both had developed an orthoselective change, this segregated as if due to mutations of genes linked to the standard markers. However, changes at more than one locus were indicated by the appearance of intermediate types in segregants; the limitation in the number of recombinants analysed does not permit a straightforward genetic interpretation (127).

Antiserum effects.—The selective action of antiserum in liquid cultures of *Brucella* has been shown to depend on the physical effect of agglutination and ensuing precipitation of cells sensitive to the specific antibodies; the availability of oxygen to the agglutinated cells is thereby decreased and selection results (15).

Interaction between mutants.—In a review of recent work, the discovery is quoted that kinetin (6-furfurylaminopurine), can mimic the selective effects of natural products of DNA breakdown in *Brucella* and *Pneumococcus* cultures (14).

Continuous cultures.—A new turbidostat has been described (54). A mathematical analysis of the theory of continuous growth, and of the conditions of stable equilibrium has been made (155). Mutation-selection equilibria were reported (42); also, conditions were defined avoiding selection of

the immunologically important, Vi-antigen-rich form of *Salmonella typhosa*, a datum of economic importance for vaccine production (52).

GENE ACTIONS AND INTERACTIONS

Chromosome Organization and Gene Action

The work by Demerec and collaborators in *Salmonella* has opened a new period in our conceptions of the relations between chromosome structure and action of genes. (36, 38 to 42, 66, 71, 72, 178, 179) This analysis was made possible by transduction technique. It owes much to the fact that transduced fragments are small but not *too* small, and hence can accommodate and transduce jointly a number of closely linked markers.

The investigation of several groups of mutants, each of which affects the same biosynthetic pathway, has given the following results. With a few exceptions (possibly including some instances of chromosomal aberrations), no two independent mutations affecting the synthesis of the same amino acid or other growth factor, are truly allelic, i.e., recombination to prototrophy is observed between any two auxotrophs for the same growth factor. However, such auxotrophs are all linked to each other. Mutants affecting the same step in a biosynthetic path, and therefore presumably forming a chromosome region [called a *locus* (38)] involved in the production of one enzyme, are all very closely linked between themselves; more so, on the average, than mutants affecting different steps. When the order of "loci," each corresponding to a given biosynthetic step, is determined from the linkage data and plotted on a chromosome map, it is found to coincide with that of the reaction chain in the biosynthetic path.

This (somewhat simplified) picture has led to the visualization of a chromosome region as an "assembly line." It seems unlikely in the light of other knowledge, that genes act directly as enzymes in these assembly lines; but the structure of the whole chromosome region involved in a given synthesis might be copied and entirely transferred to other structures involved directly or indirectly in enzymatic activity.

The assembly line theory is not new to genetics. But when it was previously put to a test no clear evidence for it could be found. Among the problems raised by these facts are the following. Does the position of genes on the map merely reflect their origin? It would rather seem that some selective advantage exists for such an order. But, if so, why is there an advantage to it in bacteria and not in fungi where genes controlling the various steps of one path are frequently, though not invariably scattered on the chromosomes almost at random. Also, none of the reasons formerly given for close linkage of genes affecting successive steps of the same synthesis—shortage, instability, or nondiffusibility of intermediates—seems important in this system. In fact, cross-feeding (syntrophism) between mutants at different loci almost always occurs.

Future refinement in techniques of analysis of genetic exchange, still largely qualitative, may add precision to the genetic picture. If tests for *cis-trans* effects (134) are made easily available, the physiogenetical aspects will

undoubtedly be clarified. At the moment, "pseudoalleles" (39) of *Salmonella* (called also "nonidentical" alleles), i.e., mutants at different "sites" (38) of the same locus cannot be compared with those of higher organisms for lack of such an analysis.

It may be useful to give a short summary of the systems so far investigated: (*a*) four tryptophan loci were detected, corresponding to the four sequential metabolic steps, X→anthranilic acid→compound B→indole→tryptophan (41). (*b*) In histidine synthesis, five loci were recognised corresponding to the sequential steps X→imidazol glycerol PO_4 ester→imidazol acetol PO_4 ester→*l*-histidinol→*l*-histidine, while a secondary chain leading to *l*-histidine from imidazol acetol ester has been described (72). (*c*) In purine synthesis, adenine and adenine+thiamine mutants were analysed (138). (*d*) Preliminary information was made available on proline, serine, leucine, methionine, and cystine pathways. An overlap between cystine and methionine markers was found, an exception to the rule of strict correspondence between biochemical and chromosomal sequences (42).

One may wonder what else can be learned from gene order in chromosomes? May chromosome maps be read, at least in bacteria, with physiologists' eyes? The linkage of Gal factors in *E. coli* is a clearly related example (124, 126). Linkage between a flagellation gene and a gene for flagellar antigens, again in *Salmonella* (105), and even the cluster of fermentation genes [Gal, Ara, Mal, Xyl, Mtl (27)] in *E. coli* are possibly not fortuitous.

Epistacy

Suppressors.—Some reversions to prototrophy in *Salmonella* auxotrophs have been shown by transduction analysis to be due to suppressor mutations. A suppressor of histidine deficiency was found to be highly specific, being effective on only one allele of one locus (156). Two suppressors of a purine requirement were also stated to be allele-specific (179). One of these purine suppressors was analysed biochemically and found to confer ability to synthesize the product of the blocked reaction (5-amino-4-imidazolocarboxamide) (66).

An analysis of the role of inhibition at the biochemical level in epistacy has been given (164).

Phase variation in Salmonella: alternating epistacy.—This well known peculiarity of the *Salmonella* system has been further explored by transduction techniques (104). In diphasic strains, cells can possess either one or the other of two flagellar antigens. Alternation from one antigen to the other takes place with a fixed probability (e.g., 10^{-3}–10^{-5}) per bacterial division. In different strains, a number of antigens are known to fall into two classes: phase 1 and phase 2. Previous analysis had shown that antigens of the two phases are controlled respectively by two loci: H_1 and H_2, independently transduced. In fact, antigens of one phase can be exchanged only with antigens belonging to the same phase group. In every cell, either the H_1 or the H_2 locus is active as expressed by the antigenic phase of that cell.

An analysis of transduction with donors and recipients in the various

phases has shown that the donor's phase can have a marked influence on the result. As phage carries over presumably only chromosomal material from the donor, the hypothesis of a purely cytoplasmic determination of the phase is excluded and the "state" (active or inactive) of the fragment transduced, as expressed by the donor's phase, must therefore be of importance. The donor's phase, is clearly important, but only in H_2 transductions. When H_1 transductions are carried out (helped by linkage of H_1 with one locus for flagellation) only the phase of the recipient determines the phase of the cells obtained after transduction.

The facts accumulated can be interpreted on the following scheme. Phase is governed by the "state" of locus H_2. If this is active, i.e., if phase 2 antigen is expressed, H_1 is inhibited; while if H_2 is inactive, H_1 can express itself and phase 1 is observed. Change of state is presumed to be distinct from ordinary mutation. The concept of "local state" may have general importance in morphogenesis.

Biochemical Studies of Some Physiological Traits

We shall limit our discussion to a few traits. The field of metabolic paths, and that of enzyme induction had to be neglected. A good general review (166) and an excellent book (168) should be mentioned.

Virulence.—Two out of four avirulent mutants of *Salmonella typhimurium* could be restored to virulence by transduction (56). Transducible avirulent mutants were adenine-dependent; after restoration to virulence, they had gained adenine independence as well, showing the connection between the biochemical defect and the behaviour in the mouse.

Biochemical mutants of the plant pathogen *Erwinia aroideae* have been analysed for their virulence to varieties of radish and turnip, and the relationship between biochemical requirements and virulence has been further documented (58). A summary of the relative roles which availability of nutrients in the hosts and presence of inhibitory substances play in determining virulence has been published (57).

Tryptophan metabolism and phage resistance.—Tryptophan requiring, T_1-resistant mutants in *E. coli* B show no simple biochemical relationship to the other known types of tryptophan dependence (67). These mutants are unique also in another respect: they are killed in the presence of the wild type, in penicillin medium not containing tryptophan. In the presence of penicillin, the wild type produces a heat-stable, trypsin-resistant substance which can kill the mutant even after both wild type and penicillin have been removed (12).

Hemin.—A peculiar streptomycin-resistant mutant in *E. coli* produces small colonies and is incapable of respiration (7). It is stimulated by hemin in a way similar to *Hemophilus.* Hemin synthesis is impaired, but ability to produce the apoenzyme of catalase is not. The apoenzyme can be extracted from cells grown in the absence of hemin, and reactivated to catalase activity *in vitro* by hemin addition. This mutant was employed (133) to investigate the importance of endogenous catalase in ultraviolet-resistance; some effect was noticed.

Photosynthesis.—A whole series of mutants affecting photosynthesis has been reported in *Rhodopseudomonas spheroides*. They fall into five groups, distinguished by the presence or absence of bacteriochlorophyll, red and yellow carotenoids, and other substances (68).

Light in the Dark.—Dark mutants were isolated from the luminous bacterium *Achromobacter fischeri*. Some synthesise luciferase normally, and can produce light in the presence of long-chain aldehydes (143).

CYTOLOGY

Only recent work on synchronized cultures will be treated. A full review on bacterial cytology (93) appeared last year, and several papers of the Symposium on Bacterial Anatomy, held by the Society for General Microbiology have analysed cytological problems of interest to the geneticist (116).

Synchronisation of cell division can be produced by several methods, of which temperature shifts are most popular. DNA synthesis is the process believed to be most sensitive to temperature change, thus making nuclear and cellular division synchronous. The following hypothesis is consistent with the observations: DNA synthesis is initiated only when its precursors have reached a certain threshold concentration; in *Salmonella* this threshold is higher at 25°C. than at 37°C. Hence, on shift to the higher temperature, DNA synthesis can start at once, accumulated precursors being now available because of the lowered threshold, and duplication of nuclei and cells follow (95). The importance of DNA synthesis in this respect is further confirmed by studies on thymineless bacteria, in which thymine starvation is known to block DNA synthesis (5). When synthesis is resumed, on addition of thymine, division is synchronised. With repeated temperature shifts, mass growth can eventually be synchronised (17).

Ultraviolet-sensitivity varies somewhat during the division cycle but no striking changes have been observed, in spite of the known ultraviolet-susceptibility of DNA synthesis. Imperfect synchronisation would, however, obscure short-lasting sensitivity peaks (18).

The cytological picture has been examined in *Salmonella* (96) and in *Bacillus megaterium* (82), using synchronized cultures; the series of changes accompanying the duplication of nuclear bodies and cell division could be clearly followed. *B. megaterium*, however, differs from *Salmonella* with regard to the series of metabolic events (129) and the stage of cell division sensitive to temperature shift. In *B. megaterium*, chilling selectively inhibits the separation of sister chromosomes, i.e., transition from "metaphase" to "telophase" (82).

TAXONOMY AND THE SPECIES PROBLEM

Systematics is giving more than one headache to bacteriologists. The Discussion meeting of the Society of General Microbiology, of which reports are available in *Journal of General Microbiology*, (Volume 12, No. 2) gives an idea of the situation.

The state of affairs will undoubtedly improve when bacterial taxonomy begins the transition from the stage of pure labelling to that of natural classi-

fication. Genetics and biochemistry are its most important tools. Genetics can supply essential information on specific differentiation. The genetic definition of species, as a group of interbreeding individuals, is important here as elsewhere (135).

Methods of study of genetic transfer in bacteria are giving full sense to the application of this definition in bacteriology. Genetic methods, may eventually be employed in the practical classification of bacteria more easily than in the classification of other living groups. This may not come about, if the number of strains not amenable to genetic transfer (i.e., strictly "asexual") is destined to remain high. Otherwise, it may be considered that genetic methods, simplified to their bare essentials, are no more exacting than standard classification methods.

The high turnover rate of microbial genetics may justify optimism in this and other cases. Thus, a statement made only a short time ago, that no bacterial group is as yet ripe for the impact of genetics on taxonomy, is already losing validity (135). The following examples should be quoted: (*a*) The *Salmonella* group. Here the current specific differentiation is not valid because practically all members of the group tested so far are potentially interbreeding. The term "serotype" is more appropriate than "species" (104). (*b*) In the *Escherichia-Shigella* group it is clear that some splitting of *Escherichia* and some lumping between *Escherichia* and *Shigella* would be appropriate. The crossability (115) and transducibility (107) between *Escherichia* and *Shigella* calls for abolition of the generic difference, at least for some of their members. The production of lactose-positive *S. dysenteriae* and worse bacterial freaks, should do more than amuse the taxonomist. (*c*) Current methods of classification are receiving some support in the *Hemophilus* group (106) where transformability is at least less easy between strains belonging to different "species."

A second path, which has recently given results that may prove of importance for taxonomy, is the biochemical analysis of cell constituents in terms of key biological constituents. Thus, the possibility of differentiating bacterial genera, and even species, on the basis of paper chromatography, has been suggested; amino acids and sugars from purified and hydrolysed cell walls were analysed (31). A better biochemical basis is being given to the differentiation between Gram-positive and Gram-negative bacteria (123). Even more interesting for the geneticist is the fact that the (adenine+thymine)/(guanine+cytosine) ratio of the DNA of 60 different bacteria has shown a clear cut correlation with systematic position; on this basis a few taxonomic changes were proposed (91). It will be interesting to see if such suggestions are further substantiated.

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GENETICS OF THE PROTOZOA[1,2]

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Species and evolution.—Sonneborn (116) has written a detailed, up-to-date treatment of breeding systems, reproductive methods, and species problems in the protozoa. Mating types, first described in 1937 in *Paramecium*, have now been found in many of the ciliates. There may be either two, or several, complementary types within each described "variety," each type capable of mating with all the others within the variety. The forms with two mating types per variety are: *Paramecium caudatum* (16 varieties); *Paramecium aurelia* (16; the exceptional variety 16 having four mating types); *Paramecium calkinsi* (2). Forms in which multiple types predominate are: *Paramecium bursaria* (6 varieties); *Paramecium trichium* (1); *Colpidium truncatum* (1); *Stylonychia putrina* (2); *Oxytricha bifaria* (1); *Euplotes patella* (2); *Euplotes woodruffi* (1); *Euplotes aediculatus* (1); and *Tetrahymena pyriformis* (9).

Recent additions to our knowledge of new mating types include Siegel's (97) discovery of mating types in *O. bifaria* and Sonneborn's (114, 116) description of *P. aurelia* varieties 10 to 16. Variety 16 was originally described by Giese as *Paramecium multimicronucleatum*, but that species is now considered synonymous with *P. aurelia* by Sonneborn because of the intermediate characteristics of certain recently discovered strains (117), one of which is called variety 12. The animals of varieties 1 to 11 and 14 are typical *P. aurelia*. The animals are small, and two micronuclei and two macronuclear anlagen are formed during conjugation and autogamy. Varieties 13, 15, and 16 are typical *P. multimicronucleatum*. They are large and characteristically develop four micronuclei and four macronuclear anlagen. Animals of the intermediate variety 12 are large like *P. multimicronucleatum*, but normally form two micronuclei and two anlagen like *P. aurelia*.

Many ciliate features, according to Sonneborn (116), represent adaptations to two different ways of life, inbreeding and outbreeding. Inbreeders typically show autogamy, much selfing, both mating types produced from each pair of conjugants, a short immature period, only two mating types per variety, many varieties, a short life cycle, good inbreeding viability, and low outbreeding viability. Examples of inbreeders are certain varieties of *P. aurelia* and most varieties of *P. caudatum*. Outbreeders show the opposite characteristics. Representatives are *P. bursaria*, *E. patella*, a few varieties of *P. aurelia*, and *O. bifaria*. *T. pyriformis* seems intermediate. The population structure of inbreeders is seen to be intermediate between that of outbreeders and haploid asexuals.

[1] The survey of the literature pertaining to this review was concluded in December, 1956.

[2] This paper covers recent publications on protozoan genetics not previously discussed in these *Reviews*.

Sonneborn concludes that the concept of the species as a genetically isolated group of individuals capable of breeding among themselves is inadequate for the protozoa because of the difficulty of identifying such groups, and because it cannot be extended to asexuals and inbreeders. He notes that irreversible evolutionary divergence is enforced by sexual isolation in outbreeders, and by genetic complexity acquired by mutation and selection in asexuals and inbreeders. He therefore substitutes irreversible evolutionary divergence for sexual isolation as the basic taxonomic criterion. He proposes that the term "species" be used to solve the practical problem of cataloguing in all organisms and suggests that it be defined in terms of "minimal irreversible evolutionary divergence that yields readily recognizable difference." The fundamental biological evolutionary unit he would call the "syngen," delimited by minimal irreversible evolutionary divergence (the notion that differences must be readily recognized is not required here). For outbreeders the syngen would be synonomous with the currently accepted, sexually isolated species of Dobzhansky and others. The syngen, however, is more general and would also be applicable to inbreeders and asexuals. In his terminology the readily recognized species *P. aurelia* would consist of 16 sexually isolated syngens (varieties, currently). It appears to this reviewer that the concept of the term species as an evolutionary unit has become too deeply ingrained to make likely the acceptance of these very practical recommendations. It should be emphasized, however, that the idea of evolutionary units of irreversible divergence among asexual organisms comparable to those of most sexual organisms is an important theoretical concept.

Recent observations have appeared on the geographical distribution of *P. caudatum* by Gilman (35) and *P. bursaria* by Chen (16), as well as studies on inviability at conjugation in *T. pyriformis* [Nanney (73); Ray (90); and Ray & Elliott (91)]. Pringle (88) has found heterozygotes of *P. aurelia* in nature, attesting to the importance of conjugation in the evolution of this species. These papers are all summarized in Sonneborn's review. Cleveland (18) has published brief summary accounts of his earlier work on the sexual cycles of flagellates of the wood roach.

Cytogenetics.—At nuclear reorganization (autogamy and conjugation) in ciliates the micronuclei undergo meiosis, while macronuclei begin degenerative changes which lead to their loss. Generally, one or more of the haploid meiotic products undergo another (mitotic) division. If the process has occurred in single animals (autogamy), gamete nuclei, consisting of two reduced sister nuclei formed at the last mitotic division, simply fuse to form a syncaryon. If the process has occurred during conjugation, one of the sisters in each conjugant migrates to the other animal of the pair and fuses with the stationary nucleus. Sometimes nuclei are not exchanged and each conjugant simply undergoes autogamy (cytogamy). A number of mitotic divisions now results in new micronuclei and macronuclear anlagen. Subsequent cell divisions restore the vegetative state which consists of one macronucleus

and one or more micronuclei per animal. Under certain conditions, fragments of the old macronucleus fail to degenerate and, instead, develop new functional macronuclei; this process is called macronuclear regeneration. Genetic as well as cytological data have attested to the regularity of these processes in several ciliates. Autogamy, common in *Paramecium*, is also found in *Tetrahymena* (20).

Genetic analysis (113) of the progeny of a single exceptional animal of *P. aurelia* which underwent autogamy led Sonneborn to the conclusion that probably two syncarya instead of the usual one were formed. One of the syncarya was derived from sister nuclei in the usual fashion and gave rise to new micronuclei and macronuclei. The other syncaryon, however, produced heterozygous macronuclei and thus appeared to have been derived by fusion of nonsister nuclei. An alternative, but less likely, explanation for the data was presented which involved differential loss of genes from macronuclear fragments during macronuclear regeneration.

Sonneborn (109) has analyzed an aberrant strain of *P. aurelia* in which micronuclei fail to produce anlagen at nuclear reorganization. Normal migratory nuclei entering this strain at conjugation produce fewer macronuclear anlagen than expected. He shows that the abnormality is due to abnormal cytoplasm and presents evidence supporting the view that the hereditary basis for the abnormality also lies within the cytoplasm. Kimball & Gaither (55) have studied a strain of *P. aurelia* having both diploid and haploid micronuclei. They show that the products of meiosis of haploid micronuclei frequently fail to form functional migratory nuclei, and fail to compete successfully with the meiotic products of diploid micronuclei in forming gamete nuclei. They present evidence that these abnormalities are under control of their own genome. Clark & Elliott (17) also find that the meiotic products of haploid nuclei in *T. pyriformis* fail to develop migratory nuclei.

The chromosomes of *P. aurelia* have been studied by Dippell (23). She finds that different stocks of variety (4) form an aneuploid series: haploid numbers ranging from 33 to 51. She notes that chromosome differences may account for F-2 inviability following interstock crosses. Ray (89) has studied the chromosomes of *T. pyriformis* and reports the haploid chromosome number of five in varieties 1, 2, 4, 5, 6, and 9. He finds (90) complete inviability in crosses within variety 5, and traces the difficulty to multispindle prezygotic divisions which lead to further nuclear abnormalities. Sonneborn (112) is unable to induce meiosis in *P. aurelia* containing micronuclei, but lacking macronuclei. He concludes that the macronucleus is necessary for the initiation of meiosis.

Mating type.—The main features of mating type inheritance in variety 1 of *T. pyriformis* have been worked out recently by Nanney and his associates (1, 72, 74 to 76). Conjugation between two types gives other types as well as the original. Thus, all the seven types of variety 1 were obtained from two clones isolated from one pond. Mating type determination is caryonidal, which means that it is determined by the macronucleus, and the macronu-

cleus is itself determined at the time of its formation during conjugation. Since the two new macronuclei, which are formed in each animal of every pair, are derived by mitosis from one diploid nucleus, they would be expected to be hereditarily identical. They are not always so, however, for their segregation to the two daughter cells at the first division following conjugation gives rise to two lines called caryonides which are often different in hereditary constitution. The parallel segregation of mating type potentialities and macronuclei at this division is the distinguishing feature of caryonidal inheritance and provides strong evidence that mating type is controlled by the macronucleus. The nature of this nuclear differentiation is unknown, but will be considered in more detail presently. The determination in each of the two nuclei proceeds independently of the other, and there is no positive correlation of the newly determined type with that of the parental conjugating animal in which the differentiation occurred.

The caryonidal determination of mating type in variety 1 gives rise not only to caryonides pure for some one of seven types, but also impure selfing caryonides which contain animals of different mating types. (No one animal, however, has more than one type.) Selfers consist of at least two mating types, and animals of different mating type are produced during vegetative reproduction. They often differentiate into pure types. However, selfing lines have been retained by selection for over 1000 generations. Among cells of a recent common origin, stabilization occurs to only one of the several types, and the rate of stabilization varies in different lines. Starvation increases the rate of stabilization. It has been pointed out that maturation of selfers can be thought of as a gradual limitation in the mating type potentialities. Reversions from the stable to the unstable state have not been recorded.

Nanney has shown that genes are important in determining mating type. Animals homozygous for *mt* II to VII may be of type II, III, IV, V, VI, or VII, but are never I. Animals homozygous for *mt* I to III, V and VI may be of type I, II, III, V, VI, but are never IV or VII. Heterozygotes may manifest any one of the seven types.

Inheritance of mating type in variety 1 of *T. pyriformis* is very similar to that in the odd numbered (Group A) varieties of *P. aurelia* (7). Thus, in variety 1 of *P. aurelia*, caryonides are determined as either type I, type II, or rarely as unstable selfers. Determination of each of the two macronuclear anlagen in a reorganizing animal proceeds independently of each other and independently of the mating type of the cell in which the reorganization is taking place. The frequency of types I and II is influenced by temperature, certain chemicals (9), and modifying genes (9). Furthermore, animals homozygous for the recessive allele *mt* I are always mating type I, but never II; while animals carrying the dominant allele *mt* I, II may be either I or II, their type being caryonidally determined as described above.

In the even numbered (Group B) varieties of *P. aurelia* (7), conjugation and autogamy usually lead to no change in mating type. If cytoplasm is

transferred at conjugation, many pairs occur in which both members become alike in type or in which one or both exconjugants give rise to selfing clones. These results would appear to indicate simple cytoplasmic inheritance. Nanney (71), however, showed that mating type inheritance in the Group B variety 4 tends to follow the caryonidal pattern. He concluded that there is macronuclear control of mating type in variety 4 also. Sonneborn (109), with an independent experimental analysis, confirmed this conclusion. Since there is a strong correlation between mating type of the newly determined macronucleus and the type of the cell in which it develops (as well as a correlation between the two sister macronuclei developing within a single animal), it is evident that the cytoplasm acts to influence the nuclear differentiation in a very specific fashion. The determining properties of the cytoplasm, however, do not appear to be directly self-perpetuating, for Sonneborn (109) has shown that the cytoplasm merely reflects the mating type potentialities of the macronucleus. Therefore, it is evident that in the Group B varieties of *P. aurelia* the newly formed macronuclei are determined by the old via the cytoplasm. Since such a determination could be accomplished by a mechanism as simple as the diffusion of some substance from old to new macronuclei, there would appear to be no need to postulate a cytoplasmic mating type-determining system with genetic properties.

The time of determination has been estimated in variety 1 of *P. aurelia* by noting that the temperature-sensitive period is just before or early in macronuclear anlagen formation (103). Estimates of the senstive period based on the effect of cytoplasm transferred at conjugation in variety 4, indicate that the nuclei may remain sensitive fairly late in their development (109). Furthermore, Sonneborn (109) finds evidence for nuclear sensitivity even before nuclear exchange. He notes in crosses of normal diploid animals of mating type VII with amicronucleate VIII animals, and normal VIII by amicronucleate VII that the mating type in the originally amicronucleate animal changes to that of the normal animal with unexpectedly high frequency. He concludes that the migrating haploid nuclei are already partially determined by the cytoplasm. Whether the changes in potentiality of the nuclei of selfers in *P. aurelia* and the gradual restriction in potential of selfers in *T. pyriformis*, result from the continued action of the nuclear differentiating mechanism, or result from segregation of already determined elements in the complex macronucleus is unknown.

Although several instances involving chromosomal changes (such as the chromosomal eliminations in *Sciara*) might be compared with these differentiations, similar cases of nuclear changes with such varied specificities are virtually unknown. Nanney (72) points out that the nuclear differentiations in amphibian development recently shown by Briggs & King (61) are, in many respects, comparable. The possibility that similar mechanisms may be involved in cellular differentiation in Metazoa makes the analysis here most significant. Unfortunately, however, little is known of the mechanisms. Hypotheses of mutation or changes in ploidy do not seem likely in view of

the facts summarized above (7). It may, however, be a bit premature to reject all notions of differences in chromosomal balance brought about by aneuploid changes during anlagen formation as the basis for the nuclear differentiations. Although the stability of nuclei during fission is hard to reconcile with such an explanation, Sonneborn's recent tests (see below under "Aging") imply that macronuclear gene doses remain rather stable during fission. The large number of types possible in some forms presents another difficult, but probably not insuperable, obstacle.

Butzel (9) suggests that the differentiations may reflect whether certain genes become functional or not. Thus, he would assume that the *mt* I allele cannot initiate the necessary reaction for type II, this reaction being controlled by the *mt* I, II allele. If the latter is present and functioning, then the animals become II; if it fails, then they differentiate as I. Beale (7) suggests that the factors determining whether genes become functional may be contained within the "macronuclear sap." Nanney (72) has further developed this concept, postulating that the intranuclear, nongenic, hereditary mechanism is a system of competing reactions resulting in a steady-state similar to that proposed by Delbrück [(21) see below] to account for antigenic inheritance in *Paramecium*. The elements are restricted to the macronucleus in variety 1 of *T. pyriformis* and the Group A varieties of *P. aurelia*, but "spill out" into the cytoplasm and affect the newly forming nuclei in the Group B varieties. It is clear, however, that all explanations at present are highly speculative and are mainly useful in suggesting new experimental approaches.

Antigens.—Many antigenic types (called serotypes) have been demonstrated in *P. aurelia* (107). Each serotype is immobilized by appropriate dilutions of homologous but not heterologous antiserum. Although changes from one serotype to another may often be brought about by changes in environment, some serotypes within a clone are constant under one set of environmental conditions, and crosses between them show a system of cytoplasmic heredity. Furthermore, it has been demonstrated that many of the serotypic differences between strains are determined by simple Mendelian factors. Varieties 1, 4, and 8 have been extensively studied, and more recent investigations now include varieties 2, 3, and 9.

Finger's work (30, 32, 33) on variety 2 shows it to be similar to varieties 4 and 8, all of which are members of Group B (see above). The fact that it is possible to isolate many different serotypes of a clone, all of which are relatively stable under one set of environmental conditions, shows that cytoplasmic hereditary influences are strong in this variety. Finger presents examples in variety 2 of two sorts of genic control first demonstrated in variety 4, a locus determining whether a given serotype can be manifested, and a locus determining specific differences in serologically cross-reacting types. He confirms the fact that antiserum against either of the cross-reacting types contains some specific antibody capable of reacting only with the homologous type and some common antibody capable of reacting with either one of the

cross-reacting types. He shows that if one assumes that specific and common antibodies combine with different reactive sites on the antigen molecules then both sites must be located on one and not separate molecules. Differences in specificity which are determined by alleles in variety 2 are typical of similar cases in the other group B varieties in that the serotypes so determined show strong cross-reactions.

The details of serotype determination have already been shown to be somewhat different in the variety 1 of Group A. Pringle's (88) recent work on variety 9 of Group A shows a pattern similar to that of variety 1; and Melechen's (68) preliminary work on variety 3, also of Group A, probably indicates similar phenomena. In all of these three varieties the number of stable types within a stock are few, and the fact that it is impossible to maintain more than one serotype of a clone for long under any single set of environmental conditions, shows that the cytoplasmic hereditary influences are weak. Thus, Pringle finds a low temperature type and a high temperature type in each stock, and finds that a change of temperature between high and low results in transformation in serotype within ten fissions. Crosses between stocks of variety 9 show that the differences in serotypes in different stocks at low temperature are determined by a series of multiple alleles at one locus, while the differences at high temperature result from alleles at another locus. In contrast to Group B, the differences in specificity determined by allelic genes in Group A are often great; usually only weak cross-reactions are shown.

The cytoplasmic inheritance exhibited by the immobilization antigens stems from the phenomenon of mutual exclusion, i.e., no more than one antigen can be manifested at a time. If mutual exclusion did not occur, then all serotype potentialities consistent with a given set of environmental conditions would be manifested and cytoplasmic inheritance would disappear. Although mutual exclusion breaks down transiently during transformation of types (5), two more instances of a somewhat more stable nature are known The first is the case of heterozygotes involving differences in specificity; here both specificities are exhibited simultaneously under appropriate environments (6). It is interesting to note, however, in a case of a heterozygote studied by Finger (33) a tendency toward exclusion could be demonstrated in the F-1 of a cross between types determined by a single pair of alleles. Margolin (66) has described a case of the simultaneous manifestation of two types within a stock. The cause of this unusual phenomenon was traced to alleles at the locus determining specificity of one of the types. The possibility that the responsible mechanism consists of an unstable state of rapid transformation back and forth between the two types has been considered, but our lack of information about the exclusion mechanism prevents testing of this hypothesis at present.

Past work has shown that transformation of serotype may be induced by homologous antiserum, changes in temperature, nature and amount of culture medium, radiation, trypsin, and chymotrypsin [for references see

Beale (7)]. Austin *et al.* (3) now report that the antibiotic patulin is effective in transforming 51D (serotype D of stock 51) to 51B. This transformation is of particular interest because it has been shown that the response is adaptive. Thus, 51B is found to be more resistant to patulin than 51D. The authors point out that this widespread antibiotic is a fungal derivative highly toxic to *Paramecium*, and consequently the adaptive response may be of real importance to *Paramecium* in nature. Antiserum-induced transformation constitutes the only other case of adaptive response which has been demonstrated, and it is, of course, important only in the laboratory.

Skaar (101) has studied the effects of past history on antiserum-induced transformation and finds effects of past nutritive condition on direction of change. Remote past history can also be of importance, the effects extending back as much as 20 days at maximum growth rate. The reason for the inertia of this change was not determined. Attempts to explain the observation that antiserum treatment may actually suppress transformation in some cases, and also the observation that change of type in other instances regularly goes through an unstable intermediate serotype, led to the proposal that transformation may be due to generalized physiological shock. This proposal would be in accord with this reviewer's data (unpublished data) on variety 2 which show that 53G at 17° C. transforms readily to 53E at 31°C. if the temperature is changed suddenly, but remains stable G at 31°C. if the temperature is raised more slowly. Similar data are reported by Beale (7). If transformation is due to shock, then a slightly different light is thrown on the adaptive response to patulin, i.e., the adaptive nature of the change may be fortuitous. Evidence that different serotypes have distinctive physiological properties is increasing, however. Differences in respiratory rate (3), in sensitivity to paramecin (2), to proteolytic enzymes (124), and of course to patulin have been noted. If many such differences do, in fact, exist, then any harmful agent capable of providing a shock may result in shift to a new serotype which may be better adapted.

Reisner (92) has obtained a series of mutants which are unable to manifest one specific serotype. All appear to be the result of mutations at the locus determining specificity of type. The results suggest a rather direct relation between serotype and gene.

Finger's (31) demonstration of the immobilization antigens by means of precipitation using an agar diffusion technique, offers a new means of studying the immobilization antigens and other antigens of *Paramecium*. He showed that of the many soluble antigens derived from homogenates and identifiable in agar diffusion precipitations, one in each serotype could be singled out as identical with the immobilization antigen of that serotype. The results show that precipitating antibodies and immobilizing antibodies are not identical, although some antibody can probably participate in both types of reaction.

Although virtually all the work on serotypes in ciliated protozoa has been on *P. aurelia*, specific antigenic types have been recently revealed in *P. caudatum* (62).

No satisfactory hypothesis has yet been suggested to account for the complex environmental, cytoplasmic, and genic control of serotypes in *Paramecium.* It is evident that when animals manifest a given serotype, there is being synthesized a specific type of antigenic molecule whose precise structure can be shown to be determined by alleles at only one locus. Transformation to another serotype results in the synthesis of a different type of antigenic molecule whose precise specificity is determined by alleles at another single locus. By means of suitable crosses, different alleles at several different serotype-determining loci may be introduced. The specificity of the allele governing the serotype currently being manifested very quickly appears in the antigens of that cell (6). Sonneborn (108) has pointed out why the plasmagene hypothesis cannot explain these facts.

Delbrück (21) has suggested that the intermediates leading to the synthesis of each serotype inhibit the reactions leading to other possible types. Hence, when one type is being formed, a steady-state is achieved which accounts for mutual exclusion. Beale (7) has discussed the improbability of this notion, and the recent work of Skaar (101) and Reisner (92), while not contradicting the idea, do not support it. Nevertheless, the conclusion seems inescapable that some sort of "feed-back" mechanism such as that suggested by Delbrück is involved. Furthermore, since so little is known of the biochemistry of such mechanisms, it seems unwise to reject them at this time. It might be noted that the first case of a complex system of alternative steady-state reactions satisfying the geneticist's criteria for cytoplasmic inheritance has only recently been reported by Cohn (19).

The hypothesis of variable gene activity (47) would also allow only one locus at a time to come to expression. It differs from Delbrück's scheme by implying that mutual exclusion is enforced by an obscure mechanism which acts at the level of primary gene action rather than at a later state of antigen synthesis.

Butzel (9), and Nanney (72) both emphasize the similarities between mating type and antigen inheritance. The main differences appear to be the greater ease of reversibility of serotype changes during vegetative reproduction and the fact that no tendencies toward caryonidal inheritance of serotypes have been reported. It should be recalled that the apparent cytoplasmic inheritance of mating types in Group B may very well involve no cytoplasmic genetic system, the cytoplasm acting only as a medium for transferring nuclear influence. It might accordingly be asked whether the same relations might not hold between the macronucleus and cytoplasm in respect to antigen inheritance. Although this possibility seems less likely because of the differences mentioned above, it is nevertheless the opinion of the reviewer that current data do not allow one to rule out this possibility completely. The mechanism controlling mutual exclusion might lie within the macronucleus, and be subject to cytoplasmic influence only during nuclear reorganization or during transformation.

Killers.—Killers of *P. aurelia* contain the genetic cytoplasmic factor **"kappa"** and liberate the poison paramecin into the fluid into which they

live (103). Mate-killers (95, 96) contain a similar genetic cytoplasmic factor called "mu," and although they liberate no poison into the medium, sensitive strains die after conjugation with mate-killers. Pi (38) is a kappa mutant, and strains bearing it show no lethal effects. Sonneborn (115) reports that none of the 119 studied strains of Group A are killers or mate-killers, while at least 30 per cent of a sample of 51 strains of Group B were killers or mate-killers when first collected. Killers had been previously known only in varieties 2 and 4, and mate-killers in variety 8. Killers have now been found in varieties 6 and 8 (115). The restriction of kappa, mu, and pi to the Group B varieties is to be explained in terms of the phylogenetic similarity between the animals of group B, and supports the validity of the A and B groupings.

Restriction of mu to variety 8 also shows that the distribution of these bodies is primarily determined by the phylogeny of the paramecia. This principle is clearly shown in the work of Siegel & Preer (98) who have studied agglutinating antigens in kappa and its relatives. In spite of the fact that the bodies of variety 2 and 4 result in similar paramecins and thus differ from the mu bodies of variety 8, the bodies of 4 and 8 show strong immunological cross-reactions and fail to show cross-reactions with the variety 2 kappas. These relations are paralleled by the fact that varieties 4 and 8 are much more closely related to each other than to variety 2.

Certain genes are known to affect the maintenance of kappa (104) and mu (64, 95). Thus, kappa is maintained at normal levels in genotype *KK*, is reduced by about one-half in *Kk* and disappears in from 9 to 15 fissions after the genotype *Kk* changes to *kk* at autogamy (11). However, Chao (12), finds that, under certain conditions, kappa retention may be considerably prolonged. Hanson (40) reports a spontaneous mutation of *K* to *k*. Balbinder (4) has re-examined additional factors originally reported by Sonneborn (105). Two loci called S_1 and S_2 (for sensitivity) have been described. Since no fundamental difference between these loci and the *K* locus is apparent to the reviewer, it is somewhat simpler to use the symbol *K* for all the loci. Thus, the original *K* gene may be designated K_1, and the new loci K_2 and K_3. In K_1K_1 k_2k_2 k_3k_3 kappa persists for 70 or more fissions and finally disappears from all lines. In K_1K_1 k_2k_2 K_3K_3 and K_1K_1 K_2K_2 k_3k_3 kappa elimination is sporadic, and occurs only in certain lines of descent. The manner of elimination of kappa in the presence of alleles at these new loci as well as at the original K_1 locus [cf. Beale (7)] appears to indicate a much more complex mechanism than the simple withholding of a substance required by the particles for multiplication. Whether the *K* alleles act to maintain kappa or the *k* alleles act to inhibit kappa is unknown; both actions are consistent with the presently available facts.

A new kappa mutation leading to reduction in maximal multiplication rate is reported by Hanson (40). He summarizes the kinds of kappa mutations thus far reported as involving changes in killing ability (22, 38, 83), reproductive rate (22), antigenic properties (38), and resistance to aureomy-

cin (25). The large-scale changes in populations of killers from strong to weak killing and even sensitivity observed by Hanson (38) and Dippell (22) emphasize the fact that studies of kappa mutation must take into account the factors of intracellular as well as extracellular ecology and selective forces. The recent work of Pittenger & Atwood (80) on *Neurospora* heterocaryons suggests that extracellular selection on the whole organism may indeed be much less important than intracellular selection of nuclei in determining population changes in that organism.

Tallan (122) has studied the process of kappa infection of sensitives, which was originally described by Sonneborn (106). Differences in susceptibility to infection among three different stocks were observed. The differences have not been completely analyzed, but were found not to arise from different alleles at the K_1 locus. Centrifugation of infectious killer homogenates reveals that the kappa-containing precipitates are much more active when combined with supernatant. Two modes of action of the supernatant might be considered: facilitation of the entrance of kappa, or enhancement of its viability or ability to multiply after entering the cell. Since kappa is about the size, shape, and specific gravity of the bacteria on which *Paramecium* feeds, large numbers must be taken into the food vacuoles, and infective particles might well gain entrance in this manner. Morphological observations on the numbers and state of kappa particles within the food vacuoles with and without added supernatant would not be difficult and might give a clue to the nature of the effect.

Little is known of the chemical nature of paramecin. van Wagtendonk (123) has reported its inactivation by deoxyribonuclease, chymotrypsin, and pepsin. Setlow & Doyle (94), however, could nor confirm inactivation by deoxyribonuclease although they found chymotrypsin to be effective. No clear explanation of the discrepancy is apparent. The reviewer (unpublished data) has also obtained negative results with deoxyribonuclease. Setlow & Doyle also show that the ultraviolet inactivation spectrum of paramecin is indistinguishable from that of protein, and quite distinct from that of nucleic acid. A word of caution might be introduced concerning the interpretation of such studies, however. It is clear that the paramecin particle is the "bright," a class of kappa particles containing a refractile body (84, 86, 87). It may well be that the actual poison is the refractile body itself or some other constituent of the "bright." In that event the "bright" might be thought of as a capsule of toxin, which would account for the apparently high activity indicated by its one-particle action. Thus, if the volume of a paramecium be taken as 2×10^{-7} cc., and if the contents of one "bright" were distributed through the contents of one sensitive paramecium, the paramecin would have an intracellular concentration of $1/2\times10^{-7}$ or 5×10^{6} particles per cc. Such concentrations are considerably higher than normally used in killer tests. Consequently, anything which disrupts the integrity of the "bright" might appear to inactivate the particle simply by diluting its contents into the surrounding medium without actually harming

the toxin. This could very well account for the apparent lability of paramecin and the failure of all efforts to isolate it chemically. Cytological observations correlated with inactivation studies should settle the question.

Previous work (99) has shown that killers of stock 51 of variety 4 respire at a greater rate than corresponding sensitives, show a reduced respiratory sensitivity to azide, and have a low cytochrome oxidase activity. Simonsen & van Wagtendonk now report (100) an active succinoxidase system present in killers but reduced or absent in sensitives. Metabolic differences between killers and sensitives of stock 51 are also shown by the fact that sensitives, but not killers, may be obtained in bacteria-free culture. It seems rather likely that considerable variation exists among different strains of *P. aurelia* in these respects. Thus, Levine (65) was unable to show respiratory differences between mate-killers and their corresponding sensitives, and the present reviewer (unpublished data) has not found differences between variety 2 killers and sensitives. Furthermore, while stock 51 variety 4 killers could not be established in bacteria-free culture with a strain of yeast and 51 sensitives could, the killers and sensitives of variety 2, together with some variety 8 mate-killers were cultivated on this medium [(98) and unpublished data].

In experiments of this type, the investigator should try to establish that strain differences are, in fact, caused by the presence or absence of kappa, and not by spontaneous hereditary changes accumulated by past mutation and selection in diverse lines. Such changes can occur; variants of altered viability are often encountered in the laboratory. It is necessary to prepare replicate killer and sensitive lines from one killer culture, maintain the lines independently and test them later for the attributes in question. A total of a half dozen lines of each type is sufficient. Although subsequent differentiation among the lines may occur, it should be at random in the killer and sensitive groups. This experimental procedure should be a standard practice in testing the physiological properties of killer versus sensitive, different mating types, or different antigenic types. Thus, the work of Boell & Woodruff (8) on a respiratory difference between different mating types of *P. calkinsi* can be seriously questioned on the basis of their failure to adopt it.

The greatest deficiency in our understanding of kappa is its unknown origin. Was it derived from some element of the genetic system of *Paramecium* or is it a symbiont derived from a once free-living progenitor? The evidence has been reviewed by Beale (7). Current data do not allow a decisive answer, but some workers feel that an external origin of kappa is the most probable interpretation of the facts currently available (111). The reviewer is inclined to this view. The question is one of phylogeny, and the basic technique of phylogeny is the tracing of relations through the similarity of related chains of forms. It is not perhaps too much to hope that the decisive links are still in existence. The several new killers in *P. aurelia* described by Sonneborn (115), the killer described by Chen (13, 14, 15) in *P. bursaria*, and the morphologically similar inclusions in other ciliates

[cf. Wichterman (125); and Fauré-Fremiet (29)] reinforce this view. A careful cytological study of many protozoa, especially the ciliates, might provide really important evidence. A new study is particularly needed because standard cytological procedures are usually inadequate for the demonstration of kappa-like bodies.

Aging.—One of the earliest genetic problems studied in the protozoa was that of the life cycle of the ciliates. Following conjugation many species show the sequence of changes exhibited by *P. bursaria* (44, 45): first, a period of high viability and sexual immaturity during which animals cannot be induced to mate; second, a period of maturity during which animals become sexually reactive; and third, a period of senescence during which growth rate declines, and increasing inviability at conjugation occurs. Conjugation prevents the onset of aging, and can even cause rejuvenescence of aged lines by producing some lines with increased viability (although some lines with even lowered viability also occur). Jennings (42) made it clear that these life-cycle changes, extending as they do over many generations, are actually changes in the hereditary make-up of the cell. The ultimate nature of the changes, however, has never been discovered.

P. aurelia was shown by Woodruff to be capable of continued multiplication without conjugation. It was inferred that the uniparental nuclear reorganization process of autogamy (which was erroneously described as endomixis) prevents death in *P. aurelia*, much as does conjugation in *P. bursaria*. Confirming earlier work (46, 102, 127), Sonneborn (110) has recently presented data on *P. aurelia* which show that in the absence of conjugation if autogamy is delayed by excessive feeding and selection, increasing numbers of animals die when autogamy is induced by starvation. If autogamy is prevented, death finally results. Progressive changes in fission rate, ability to mate, and tendency to undergo autogamy are also observed. Autogamy prevents these changes and is thus comparable to conjugation in its effect on aging, as assumed by Woodruff & Erdmann (128).

New attacks on the aging problem have made some progress. Mitchison (70) concluded that aging in *P. aurelia* cannot be accounted for by micronuclear gene mutation. Sonneborn & Schneller (119) conducted a breeding analysis involving young and aged clones of variety 4 of *P. aurelia*. They showed that death at autogamy in old clones is caused by chromosomal aberrations in the micronuclei. For example, crosses between young and old clones produced vigorous hybrids which showed much inviability at the subsequent autogamy. These results, together with cytologic observations by Dippell (24), indicate that the death at autogamy observed in aging is due to frequent chromosomal aberrations in the micronuclei. Such defects are manifested by variable numbers of micronuclei in old lines, clumping of chromosomes, unequal chromosome segregation, inability to form normal macronuclear anlagen, and other cytological abnormalities.

Sonneborn & Schneller (119) find, however, that chromosome aberrations are a secondary manifestation of aging, not the primary one. The ob-

served aberrations do not arise in a simple spontaneous fashion: when successive autogamies occurred about every 25 fissions, inviability at autogamy was about 0.7 per cent, yet 100 per cent inviability was obtained at autogamy when lines were 250 fissions old. The aberrations arise in high frequency in old lines, not in young. Sonneborn & Schneller (120) also considered the question of whether the effects of parental age are transmitted through autogamy. They tested viability at autogamy in lines with short and long interautogamous intervals, and obtained no cumulative effects; old lines were restored to youth by one autogamy. They noted that at macronuclear regeneration lines of both higher and lower vigor are produced but these also eventually senesce.

As indicated above, the nature and even the seat of the primary deleterious changes responsible for aging and death at nuclear reorganization are unknown. Many theories have been proposed to explain the phenomena (43, 110), and two of these have been examined recently. Sonneborn and co-workers (120, 121) have made an attempt to test the suggestion of Fauré-Fremiet that aging results from chromosomal unbalance brought about by amitotic division in the highly polyploid macronucleus. [The cytological evidence for a polyploid macronuclear structure has recently been reviewed by Grell (36)]. Macronuclear regeneration was obtained in aged lines heterozygous at several different loci. The resulting lines showed variation in their phenotypic expression of the traits in question. Since only one allele at each locus, and always the same one, failed to be expressed, it was concluded that the variations were due to genotypic expression, not gene dosage. Kimball & Gaither (54) have tested the hypothesis that reduced fission rate is due to macronuclear mutation. Animals of *P. aurelia* were given an x-ray dosage of 80,000 r. The rate of fission during aging in clones derived from these animals did not differ from that of a control group receiving no radiation; fission rate in both declined at the same rate. It would thus appear that this hypothesis is invalid.

Radiation effects.—Four harmful effects of radiation on cells are: (*a*) fission delay; (*b*) immediate death; (*c*) delayed death; (*d*) death at the next sexual reproduction. It is of interest to inquire whether these effects are due to genetic or nongenetic damage. In spite of the fact that damage leading to fission delay has recently been shown by Ord & Danielli (79) to be nuclear in *Ameba proteus*, such delay persists for only a few fissions and is therefore not hereditary. In *P. aurelia* the delay does not last more than eight fissions (59, 82). Immediate death would not be expected to result from mutations or chromosomal aberrations (51).

The nature of delayed death is not so clear, however. Death due to gene mutations and chromosomal aberrations should be frequent in haploids, rarer in diploids, and correspondingly less frequent in forms having higher levels of ploidy. The site of delayed death induced by x-rays and nitrogen mustard has been studied by Ord & Danielli (77, 78, 79) in *A. proteus*, a form thought to be highly polypoid. They utilized the technique of nuclear transfer and concluded that although the site of the damage was usually

nuclear, high doses induced reversible cytoplasmic damage, while still higher doses induced irreversible cytoplasmic damage. It was also shown that reversibly damaged cytoplasm could damage nuclei. It is tempting to conclude that death from damaged nuclei is the result of dominant lethal mutations and chromosome aberrations. But this interpretation is open to doubt in view of the apparent high level of ploidy and the fact pointed out above that noninherited fission delay in *Ameba* has a nuclear basis. It should be noted that the general concept of dominant lethals is not quite as secure as that of other genetic factors. In considering hereditary variations in general, we require that they be transmitted to future generations; this useful criterion of a genetic effect cannot be applied in the case of the dominant lethal mutation. Kimball (51) concludes that delayed death in *Paramecium* (whose functional macronucleus also displays a high level of ploidy) is not due to mutation or chromosomal aberrations.

Death at sexual reproduction, many cell divisions after radiation, clearly has an hereditary basis, however. Death at conjugation and autogamy has been extensively studied in *P. aurelia*. Kimball & Gaither's recent work (56) shows that the site of x-ray damage leading to death at sexual reproduction is nuclear and not cytoplasmic. Crosses of normal animals to irradiated and unirradiated amicronucleates showed no lethal effects resulting from the radiation. Furthermore, most of the results from autogamy and various types of matings can be accounted for on the basis of recessive and dominant lethal or deleterious mutations. One or two instances, however, have not been satisfactorily explained (49). In spite of these few cases, the amount of death following autogamy seems to provide a valid index of genetic damage to nuclei. Kimball's finding (48) that fractionation of β-radiation gave the same effect as a single dose, shows that "two-hit" aberrations are not important. The main effects are thus presumably gene mutation and single breaks. The incidence of dominant lethals appears to be low (56) and the results suggest that most of the reduced viability arises from the action of a number of recessives, each with a small but cumulative effect (48).

Various factors have been shown to influence x-ray-induced postautogamous death in *P. aurelia*. Kimball (52) studied the sensitivity to radiation administered during different parts of the mitotic cycle, and found that sensitivity was greatest immediately after mitosis and during the first two-thirds of the interfission interval. During the last third of the period and during all of mitosis resistance was several times as great. It is suggested that the time of change from sensitive to increased resistance coincides with the time of reduplication during interphase.

The well-known "oxygen effect" has been reported in *P. aurelia*. Kimball & Gaither (53) have shown that post-autogamous death after x-irradiation is greater in oxygen or air than in nitrogen or helium. Furthermore, recent work (50) demonstrates that exposure to the different atmospheres is effective during, but not before or after, the period of radiation.

Certain postirradiation treatments reduce death at autogamy. Kimball, *et al.* (57, 58) report that reduced temperature, starvation, streptomycin,

and H_2O_2 are effective after x-irradiation. Temperature had only a slight effect, however; and in the experiments of Powers (82), no significant differences were observed. Kimball *et al.* report that in some experiments streptomycin had the reverse effect, autogamous death being stimulated. The situation appears complex, with the variable post-autogamous death being paralleled by variations in sensitivity of the paramecia to the antibiotic during exposure. No treatments were effective after the first postirradiation fission.

The results with H_2O_2 are of particular interest because of the hypothesis that part of the mutagenic action of x-radiation is due to the liberation of H_2O_2. It has also been suggested that the oxygen effect may be in part due to the increased production of H_2O_2 under high O_2 tensions. Hearon & Kimball (41) were able to estimate the concentration of H_2O_2 within the nucleus during radiation and during exposure of animals to H_2O_2 solutions. These estimates were based on diffusion theory and measurements of catalase activity of paramecia. Kimball *et al.* (60) found that exposure of nuclei to H_2O_2 in equal and higher concentrations equal to or higher than those produced by x-radiation was ineffective as a mutagen and ineffective in enhancing the mutagenic action of x-rays. This was so even for the very high nuclear H_2O_2 concentrations obtained when catalase was inhibited by cyanide. Thus, a role of H_2O_2 in x-ray mutagenesis and also in the oxygen effect in paramecium is disproved.

Mitchison (70) has studied ultraviolet-induced postautogamous death, and concludes that some but not all of the lethal affects are due to micronuclear gene mutation. His aberrant cases are possibly explained by effects on nuclear behavior described by Sonneborn *et al.* (118). By using suitable markers, these workers could show that in crosses between irradiated and normal animals, the irradiated animals often fail to contribute a migratory nucleus and may have both migratory and stationary nuclei rendered nonfunctional. As a result, haploidy (sometimes followed by diploidization), macronuclear regeneration, and cytogamy, may follow. Normal nuclei received by an irradiated mate during conjugation may fail, presumably as a result of radiation-induced asynchrony in the events of conjugation. Kimball & Gaither (56) have performed similar experiments using x-rays. They confirm these results except that they find no evidence for asynchrony. They also describe haploids arising at conjugation from one-way transfers of genes, which result from lack of formation of migratory nuclei by the irradiated mate. Similar behavior leading to haploidy was reported in *T. pyriformis* by Elliott & Clark (17, 26). Kimball & Gaither (55) report that in crosses of a normal diploid with a clone containing one diploid nucleus, products of the diploid nucleus had a strong competitive advantage over those of the haploid for surviving to become the functional gamete nuclei. Radiation interfered with these events by removing this advantage, making products of the diploid nucleus much less likely to contribute to the next generation. It seems likely that part of the interference is due to chromosomal aberration, since conjugation between diploids and haploids reveals similar interference

attributable to grossly aberrant meiotic segregations. The rarity of dominant lethals, shows, however, that the x-ray-induced effects on nuclear behavior are probably not altogether a result of chromosome aberrations; and an unknown mechanism is therefore postulated.

Hanson (39) has specifically damaged one of the two gullets in double animals of *P. aurelia* by use of an ultraviolet microbeam. Regeneration of damaged gullets sometimes occurred; but animals so treated often reorganized as singles. Although it is not clear how long such radiation-induced singles were followed, permanent loss of the gullet would indicate that it has a self-reproducing hereditary basis which can be destroyed by radiation.

Strain differences.—A number of naturally occurring and induced variations has been reported. Schoenborn (93) finds that frequent, spontaneous changes in growth rate occur within clones of *Astasia longa;* their genetic basis is unknown. Miller & van Wagtendonk (69) correlate the chromosome number of different strains of *P. aurelia* with population density in bacteria-free culture. Powelson (81) has studied kinetosomes in strains of *P. aurelia* and finds that the numbers in certain regions are constant within a strain but differ between strains and varieties. Finley & Williams (34) find differences in ninhydrin-positive paper chromatographic patterns between asexual and sexual stages in *Vorticella microstoma.* Lee (63) compared *P. aurelia*, *P. caudatum*, and *P. multimicronucleatum*, and found no differences in ninhydrin-positive bands, but did find characteristic differences in fluorescent bands. Butzel & Martin (10) found no differences in amino acids between strains, mating types, and varieties of *P. aurelia.* Levine (65) finds two strains belonging to two different varieties of *P. aurelia* qualitatively similar with respect to 30 enzymes. Elliott & Outka (28) report naturally occurring strain differences in *T. pyriformis* in ability to ferment levulose, lactose, and galactose, and Elliott (27) also finds differences in ability to grow without pyridoxine and serine. Preer (85) reports a naturally occurring, temperature-sensitive strain of *P. aurelia*, differing from normal by a single gene. Wichterman (126) reports an unsuccessful attempt to select a radiation-resistant clone of *Paramecium.* Hall (37) has obtained sulfonamide-resistant lines of *Chilomonas paramecium*, but it is not stated whether the resistance is inherited. An interesting case of adaptation to KCN in *T. pyriformis* is described by McCashland (67). His conclusion that mutation and selection are not involved seems justified. However, the fact that deadaptation requires many generations (75 or more) is reminiscent of Jollos' *Dauermodificationen* and suggests to the reviewer that the phenomenon has an hereditary basis. In view of the action of cyanide on the cytochrome system, it might be that the changes are similar to the cytochrome mutants of yeast and *Neurospora.*

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THE HOMOGRAFT REACTION[1]

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Introduction

Homotransplants are transplants between genetically dissimilar individuals or, more specifically, from one inbred strain to another unrelated inbred strain. *Isotransplants* are transplants within a genetically uniform inbred strain. Typically, isotransplants will grow, but homotransplants will not. The consistency of this behavior of transplants within, as compared with between, inbred strains has made such strains, and particularly the large variety of highly inbred strains of mice, one of the basic tools of investigation in this area. Transplantable tumors, because of the ease and rapidity with which subcutaneous or intra-abdominal transplants can be performed, are also a valuable research tool, though normal tissue grafts are the preferred instrument for many purposes.

The reaction of a host to a graft of normal or tumor tissue from a genetically dissimilar individual, typically leading to ultimate graft rejection, is known as the *homograft reaction*. Hosts receiving two successive grafts from the same, or genetically related, donors, usually reject the second more rapidly than the first. In the case of tumors, the first implant may grow to large size before regressing, the second not at all. The reaction to a second graft has been called the "second-set response."

A number of reviews have appeared dealing with immune phenomena in relation to tissue grafting. Three of the most recent are written by Eichwald (1), by Rogers & Converse (2), and by Gorer (3). The last named is recommended as particularly thorough, critical, and up-to-date. Two additional papers which are relevant deal with transplantable tumors (4, 5). The first is a general review of the field, the second discusses the susceptibility of tumor cells to humoral antibodies.

We shall discuss here only certain selected aspects of the homograft problem. Some of the material covered by Gorer (3) which it would otherwise be pertinent to include, will be omitted. We shall not deal with the

[1] The survey of the literature pertaining to this review was concluded in February, 1957.

[2] Of the material herewith summarized, studies by the author and co-investigators were supported by a research grant C-1329 from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service, by a grant from the American Cancer Society, and by a grant from the Anna Fuller Fund.

I am indebted to Dr. Nathan Kaliss, Dr. Andrew Kandutsch, and Dr. Henry Winn for the stimulus provided by many interesting discussions of the homograft reaction, and to Drs. Kandutsch and Winn for a critical reading of the manuscript of this review.

problem of actively acquired tolerance (6), although this is one of the very important recent developments in the field.

Histocompatibility Genes

Experiments with the mouse have demonstrated the existence of certain genes or genetic loci which play a vital role in the acceptance or rejection of grafts. The genes which play this role have been called histocompatibility genes (7). Estimates place the number of loci at not less than 14 (7, 8); the number of genes, including all allelic or alternative forms at one locus, is many times this number.

The rule governing the action of these genes is that an incompatibility reaction results when any one or more of the histocompatibility alleles present in the donor is absent in the host. The reaction may not be strong enough in all cases to cause graft rejection but, except for grafts in certain protected situations such as the anterior chamber of the eye, it is probably always present. This relation between the occurrence of a homograft reaction and the presence of something foreign in the graft, presumably a gene-determined isoantigen, is immediately suggestive of an immune-type reaction. Also apparent is a close parallel to the genetic determination of blood groups and transfusion reactions.

The detailed analysis of histocompatibility genes requires technical procedures which have so far been applicable only in the mouse. It would be out of place to attempt to describe the methods here, but a brief summary of our current knowledge of these genes is necessary for an understanding of various facts in connection with the homograft reaction. For further information the reader is referred to various reviews (4, 7, 9, 10).

Four loci, *H-1* in linkage group I, *H-2* in linkage group IX, *H-3* in linkage group V, and a Y chromosome locus, have been identified (11 to 16). *H-2* is a complex locus with at least twelve alleles, each characterized by six or more antigenic components or factors (10, 12, 17, 18). *H-1* and *H-3* are less completely analyzed, but there is evidence for the existence of four alleles at *H-3* (19). *H-2* is a "stronger" locus than *H-1* or *H-3* in the sense that whereas grafts, particularly tumor grafts, will frequently grow successfully when donor and host differ at *H-1* or *H-3*, they will seldom do so when the difference is at *H-2*. (Certain so called nonspecific tumors are an exception.) This difference in the behavior of grafts is probably a direct outcome of the fact that *H-2* determines an isoantigen capable of evoking a strong immune response, whereas *H-1* and *H-3* determine much weaker antigens; in fact *H-2* seems to hold a position of quite unique importance in the mouse in this respect (10, 20, 21).

We have suggested elsewhere (5) that the isoantigens determined by histocompatibility genes are probably present in the cell membrane. The most direct evidence for this is the fact that isoantibodies, or at least *H-2* isoantibodies, are absorbed by intact cells. We may well suspect that they are also a part of the intracellular membranes so beautifully demonstrtaed by the electron photomicrographs of Palade and others (22, 23).

Histology of the Homograft Reaction

The histology of the homograft reaction has been the subject of numerous studies going back over many years (4). We shall confine ourselves to a brief summary of a recent investigation by Darcy (24) based on intradermal implants of submaxillary glands in the rabbit. Homografts were removed by biopsy at 4-day intervals and sectioned, with corresponding autografts as controls. Counts and a detailed analysis were made of invading cells.

The surface of both autografts and homografts was vascularized and apparently healthy on the fourth day. On the eighth day homografts were invaded by numerous lymphocytes and mature and immature plasma cells, and the blood vessels were greatly dilated and engorged; on the twelfth day the vessels were largely broken down and graft destruction was well underway; by the sixteenth day it was complete. Autografts progressed normally during this period without pronounced invasion of host cells.

Darcy also investigated the reaction (second-set reaction) to the second of two groups of grafts made 24 days apart from the same donor. The points of principal interest were a pronounced invasion of lymphocytes as early as four days, and more rapid destruction of the whole graft. We shall return again to certain details of this study.

Humoral Antibodies Produced by Homografts

In a classic paper published in 1937, Gorer showed that the rejection of a tumor homograft resulted in the formation by the host mice of antibodies capable of agglutinating red cells from the inbred strain of the donor (25). This was the first clear proof of an immune reaction to homografts. This result also constituted evidence that the red cells of the donor strain and the tumor shared an antigen in common. It was also shown by grafting and blood-typing mice of F_2 and backcross generations that this common antigen, called antigen II, segregated in good Mendelian ratios and hence was gene-determined. In a subsequent study, Gorer, Lyman & Snell (26) related this to a gene in linkage group IX which was therewith called histocompatibility-2 or *H-2*. Improved methods of red cell agglutination adapted to the mouse which have been developed by Gorer and co-workers (27) are the basis of most of our information about the antigenic factors or components associated with the various *H-2* alleles.

Attempts to identify red cell agglutinins in the sera of mice immunized with tissues which introduced foreign *H-1* or *H-3* antigens, but not *H-2* antigens, have failed (19). However, the principal that graft rejection is accompanied by the formation of humoral antibodies directed against the tissues of the donor has been extended to skin grafts in mice, rabbits, and man (28, 29, 30) and to bone grafts in dogs (31). Woodruff & Forman (32) have shown that the serum from rats receiving homografts of either lymph nodes or skin produces a lymphocytopenia when injected into the donor strain. In general, the titer of *H-2* red cell agglutinins in mice which have rejected a skin graft is low compared with the titer in mice which have rejected a tumor graft. Multiple tumor grafts are capable of evoking a very high titer.

The Cellular Factor in Homograft Rejection

There are studies going back over a number of years which point to some factor inimical to donor tissues in the lymphoid cells of animals which have regressed a graft (3, 4). It was the investigations of Mitchison and co-workers (33 to 35) that first clearly defined this factor, and we shall confine our discussion largely to their work.

Mitchison's approach was based on the observation that immunity to homografts in mice can be transmitted to other mice by transfer of the lymph nodes draining the site of a graft. Mice receiving immune nodes intra-abdominally give a second-set response to a tumor homograft given subcutaneously at the same time. The immunity can be passively, or, to use the phrase of Billingham *et al.* (36), "adoptively" transferred. The term adoptive is appropriate because the transferred lymphoid cells continue to function in or are adopted by the new host. In this connection it was one of the basic tenets of Mitchison's approach that the lymph node transfer was an isograft, made within an inbred strain. The tumor used for immunization of the original host and for challenge of the secondary host was a homograft, coming from a foreign strain. The measures of immunity employed were the relative weights of tumors in test and control mice at a fixed interval after implantation, or the percentage of tumors still viable as determined by return of remaining tumor tissue to the native strain.

When the tumor was implanted subcutaneously on the right side, only the regional lymph nodes receiving lymphatic drainage from this side were able to transfer immunity. The contralateral nodes were inactive, as were frozen-thawed nodes, and also the spleen. Attempts at passive transfer with antiserum were likewise unsuccessful. It was necessary to use the regional nodes in the proportion of four donors per host to achieve maximum protection. The possibility that the lymph nodes were transferring living tumor cells or isoantigens from the tumor was considered and in all probability ruled out by ingenious experiments.

By transferring lymph nodes at different intervals after the immunizing tumor implant had been administered, the time of appearance and disappearance of the immune factor was studied. Nodes taken from mice three days after tumor grafting were inactive. Activity was at a maximum at five and at ten days, but again absent at fifteen and twenty days. The immune factor thus reaches an early peak but is of short duration. Red cell isoagglutinins evoked by the same graft come to a peak later and persist longer. These relationships are represented in Figure 1A. While the precise shape of the two curves in this figure is open to question, the features of their relative form mentioned above would seem to be adequately established.

In the case of the secondary response to a tumor homograft (Fig. 1B), note that whereas the hemagglutinin titer is increased, the rise in cellular immunity, as measured by the capacity of transplanted immune nodes to kill grafts, is less following a second graft than following the first. It should be emphasized that the measure of cellular immunity is not quantitatively

precise so that the shape of this curve is an approximation only. The rising portion of the curve showing hemagglutination titer may also be subject to considerable error.

Hosts receiving isologous immune nodes or spleen developed hemagglutinins in their serum. The time relationships of this phenomenon were

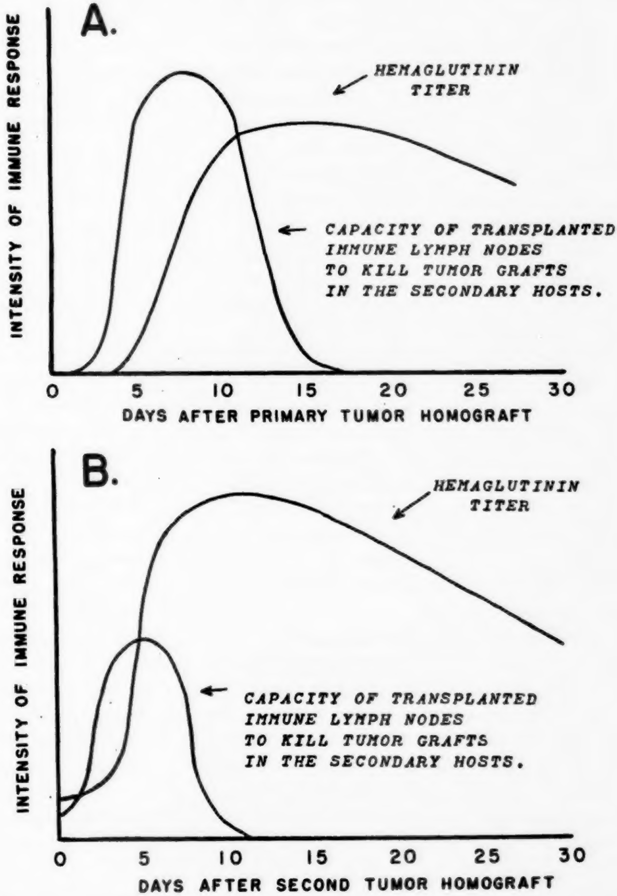

FIG. 1, A and B. Diagramatic representation of the development of humoral and cellular immunity in mice with tumor homografts. Based on data of Mitchison and co-workers (31 to 33) A. The response to a primary graft. B. Secondary response to the second of two implants of the same tumor.

studied but will not be detailed here. We may note however that the relative capacity of the transferred spleen, as compared with the nodes, to produce antibody increased with the interval between immunizing tumor implant and transfer.

A series of experiments, too complex to be detailed here, using stocks of known histocompatibility genotypes, served to demonstrate the specificity of the phenomenon and the importance of the *H-2* system of alleles.

A significant role in graft rejection of an immune factor bound to living lymphoid cells is indicated. The parallel to delayed or tuberculin hypersensitivity, which is also mediated through cells (37), is apparent. We shall revert again to Mitchison's work, but meantime turn to other studies emphasizing the role of cells in transplant immunity.

Brncić, Hoecker & Gasić (38) reported transfer of immunity by means of spleen, lymph nodes being much less effective. Their experiment involved a number of unusual features and there is some uncertainty as to the reason for the difference from Mitchison's results, but the route of immunization may have been the determining factor.

Kidd (39) showed that incubation of lymphosarcoma cells with minced immune lymph nodes for 1 to 2 hr. before implantation abolished the capacity of the tumor to grow. He also carried out a cytological study of tumor homografts. Lymphocytes appeared at five to seven days, and thereafter were found in intimate contact with dying cancer cells. In regions where the lymphocytes had not penetrated, the tumor cells continued to proliferate. Weaver, Algire & Prehn (40) investigated the growth of transplantable tumors in diffusion chambers, permeable to body fluids but not cells, placed intra-abdominally in mice. In a variety of combinations of immune and nonimmune hosts, it was found that tumor homografts were killed rapidly if, and only if, the diffusion chamber also contained pieces of immune spleen. The presence of nonimmune spleen, foreign to the tumor, led to ultimate but much delayed damage.

These studies point to an intimate interaction between immune lymphocytes and target cell. On the basis of cytological observations, Weaver *et al.* concluded that immunized lymphocytes died in attacking target cells. These authors find a precedent for this observation in the demonstration by Miller *et al.* (41) that normal human leukocytes are lysed in the presence of tuberculoprotein, the heat labile globulin from tuberculous plasma, and complement. Darcy (24) also noted "the curiously intimate penetration of the homograft epithelium by lymphocytes." Darcy questioned the significance of the lymphocyte in graft destruction, yet one of his reasons for this, a relative decrease in lymphocyte concentration in grafts undergoing especially rapid destruction, is quite in accord with the concept that the lymphocytes must disintegrate in the process of destroying their target cells.

We now may ask what role, if any, humoral antibodies play in the homograft reaction. To answer this question we need first to consider a phenomenon known as the enhancing effect.

The Enhancing Effect

The enhancing (or XYZ) effect is an increased growth of tumor homografts in animals which have been pretreated with one or more injections of nonliving donor tissue. The treatment is such that one would expect immunity, but the response is exactly the opposite. Under favorable conditions the response is striking—all or nearly all treated animals succumb to progressively growing tumors while all controls survive (42, 43).

The tissue used for pretreatment can be either freeze-dried (lyophilized) tumor or tumor extracts (44), or preparations of any one of several normal tissues of which spleen is probably the most effective (45). It need not come from the same inbred strain as the tumor used for homografting, but to produce more than a minor effect it must share with the tumor *H-2* antigenic components which are lacking in the host. On the basis of these specificity relationships, it was concluded that the enhancing substance (or the principal enhancing substance), is identical with the *H-2* antigen (46).

An important step in elucidating the phenomenon was taken when Kaliss and co-workers (47, 48) demonstrated that the serum of mice that have been given the enhancing treatment will passively transfer the effect. By using serum fractions and by other appropriate tests a strong presumption was established that the active agent is an antibody. This is in accord with the evidence mentioned above that the (principal) enhancing substance is the *H-2* isoantigen. Antiserum prepared in rabbits against donor tissue is also effective.

Kaliss (49) has further demonstrated that antiserum from mice which have been immunized with living tumor will cause enhancement of the growth of that tumor in secondary hosts. The original hosts would of course have resisted a second inoculation of the tumor, so that this experiment beautifully separates the enhancing and the immune factor (for a qualification and further discussion of this statement, see below).

Both Gorer (50), and Mitchison & Dube (34) have shown that mice injected with foreign-strain, freeze-dried tissue develop isoagglutinins reacting with the red cells of the donor strain, though to a lower titer than that evoked by living tissue. It is at present undetermined whether the agglutinating antibody and the enhancing antibody are one and the same.

A further important contribution has been made by Kandutsch & Reinert-Wenck (44) in a study of the chemistry of the enhancing substance. The necessity of using a somewhat laborious biological test system which is not quantitatively sensitive imposes limitations which nevertheless have not prevented the attainment of substantial results.

Four principal fractions were prepared from tumor homogenate and submitted to various forms of analysis. Fraction 1 was an acid-soluble fraction extracted from cytoplasmic elements which had been separated from nuclear material by preliminary centrifugation. Fraction 2 was an acid-insoluble fraction of the cytoplasm containing most of the ribonucleoprotein (RNAP) of the tissue. Fraction 3, predominantly deoxyribonucleoprotein was extracted

from a low-speed sediment (nuclear material) with 1*M* NaCl and purified by precipitating four times in 0.14 *M* saline. Fraction 4 was a washed insoluble residue. All fractions were tested for DNA, RNA, hexosamine, lipide, and assayed for enhancing activity at different dose levels in 30 mice. Most tests were repeated several times.

All fractions were active. Since fraction 1 lacked DNA this was effectively ruled out as the active agent. Moreover, activity was not destroyed by treatment with deoxyribonuclease, nor by hyaluronidase nor trypsin. In various preparations of ribonucleoprotein and deoxyribonucleoprotein subjected to additional purification, there was no relation between activity and the concentration RNA or DNA, but hexosamine was always detectable in significant amounts. Lyophilized whole tumor was still active after rigorous extraction of lipides, indicating that these were not the active agents.

The significance of hexosamine was further emphasized in these studies by the rapid destruction of activity with dilute solutions of sodium periodate. Activity was also destroyed by exposure to 50 per cent urea, to 90 per cent phenol, or to solutions more acid than pH 5 or more alkaline than pH 9.

The results are interpreted as indicating the presence of both carbohydrate and protein in the structure of the active substance. The substance may thus belong to the class of compounds known as mucoproteins, as do some of the blood group antigens in man. This is compatible with other evidence pointing to the *H-2* antigen as the (principal) enhancing substance.

Green & Wilson (51) have reported the production of enhanced growth of a rat tumor by pretreatment with lipide tumor fractions. It should be noted that Kandutsch & Reinert-Wenck (44) obtained temporary stimulation of growth with a lipide fraction, but not the progressive growth characteristic of enhancement. Horn (52) has found accelerated growth of the Ehrlich ascites tumor in mice pretreated with isolated tumor nuclei lysed in distilled water. To this reviewer, it appears misleading to call this preparation a "nucleoprotein" since, as Horn specifically states, it certainly contained a variety of other materials. Nuclear membranes and quite probably fragments of the cell membrane (53) must have been present, so the effect can have been due to the same sort of carbohydrate-protein complex to which Kandutsch and Reinert-Wenck attribute the activity of their preparations.

How general a phenomenon is the enhancing effect? Tests made with a variety of tumors (54) show that not all tumors respond to pretreatment of the host with enhanced growth. In fact, two leukemias were found to give the opposite response. The presumption is that these tumors were inhibited instead of enhanced by antibody. I have reviewed elsewhere (5) the published evidence as to the differences in the response of different tumors to humoral antibody, and it would be out of place to repeat this material here. Suffice it to say that some transplantable tumors, including many and perhaps most leukemias, seem to be susceptible to a cytotoxic effect of antiserum in a way that other tumors are not. Another group of tumors responds to

antiserum by enhanced growth, still others may be largely unresponsive.

We have so far spoken only of studies of the enhancing effect carried out with tumor homografts in mice, but the phenomenon is not limited to this test situation. Some of the earliest investigations were carried out with rats and rabbits [reviewed in (43)]. A similar phenomenon which has been called the accelerant effect has been noted in experiments with isografts by Casey and co-workers (55, 56) and by Bittner and co-workers (57, 58). Evidence that the accelerant factor is a lipide (58) may point to important differences from the enhancing effect, although, as already pointed out, pretreatment of the host with lipide extracts of a tumor may enhance the growth of the tumor when homografted.

Pikovski & Schlesinger (59, 60) have obtained progressive growth of mouse tumors in rats by giving repeated injections of freeze-dried donor tissue starting at an early age.

Of particular interest is the question whether the enhancing phenomenon is demonstrable with homografts of normal tissue. Transplantable tumors have a growth potential not possessed by their normal counterparts, so that it is altogether possible that they will respond to alterations in the host too slight to affect the growth of other types of homografts. Nevertheless several investigators have now reported some measure of success with normal tissues. Prolongation of survival of skin homografts in rabbits or mice, or both, has been reported by Allen *et al.* (61), by Billingham *et al.* (62), and by Hardin & Werder (63). Parkes (64) obtained similar results with ovarian homografts in rats. All these authors except Billingham *et al.* used for pretreatment extracts or homogenates of the tissue or organ to be grafted, so prepared that living cells were presumably not present. The materials used for pretreatment by Billingham *et al.* were lyophilized liver, kidney, or spleen from the same inbred strain of mice that provided the skin for grafting. Billingham & Sparrow (65) also succeeded in increasing the survival time of skin homografts in rabbits by a factor of 2 or 3 by prior intravenous injections of living dissociated epidermal cells from the graft donor.

Of the various investigators who have attempted enhancement of the survival of normal tissue grafts, only Hardin & Werder (66) have reported permanent survival, although Parkes has noted the survival of ovarian grafts for at least three months. Negative or equivocal results were obtained by Kaliss (67, 68). There are a number of unexplained features of Hardin and Werder's results. They were most successful with skin grafts made within the noninbred CFW strain, the material for treatment being an extract of pooled skin. Such a tissue pool prepared from a partly inbred strain may have been presumed to share the isoantigens of most grafts. Possibly there has been sufficient inbreeding in the CFW strain so that donor and host did not usually differ at the "strong" *H-2* locus, although this is entirely speculative. Their report of one-year survival of a graft from a BALB/c to a C3H mouse (method of treatment not specified) is without precedent, and their success in one experiment with injections given after grafting is also unusual.

Allen *et al.* (61), who worked with rabbits, were careful to use, for any given host, the same donor to provide both skin extract and skin for grafting, a precaution necessary when noninbred animals are used. They also tested each injected and grafted rabbit, after graft sloughing, for sensitization by an intravenous injection of the appropriate extract. All animals died of anaphylactic shock, which may be taken as evidence that humoral antibodies were present. Billingham & Medawar (69) have objected to their results on the ground that they added phenol to their skin extract, and report prolongation of survival of skin homografts in rabbits by pretreatment with phenol alone. Only future studies can resolve this uncertainty, but the susceptibility of the rabbits used by Allen *et al.* to anaphylactic shock is evidence that their treatment was antigenically effective. Experiments conducted with rabbits, because they are genetically heterogeneous, are often difficult to reproduce as Billingham *et al.* (62) themselves have found.

Aside from the one test for the presence of antibody made by Allen *et al.* (61), none of the studies of enhancement in normal tissue grafting has included tests of the role, if any, which antibody may have played. Until such tests are forthcoming, the closeness of the parallel to the enhancing effect as demonstrated with transplantable tumors cannot be accurately assessed.

The Mechanism of Enhancement

While a positive answer cannot yet be given as to the reason why treatment of the host with nonliving donor tissue prolongs the life of a subsequent homograft, it is noteworthy that Snell (70), and Billingham, Brent & Medawar (62) have simultaneously proposed the same explanation. It is of course possible that more than one mechanism is at work; the explanation proposed by Snell and Billingham *et al.* concerns the role of antiserum.

Briefly, the suggestion is that antiserum prevents or delays the antigens of the homograft, or at least effective antigens, from reaching the regional lymph nodes. The nodes are thus unable to generate the cellular immune factor which is the principal agent in graft destruction. Snell has called this a "walling-off" of the graft, and Billingham *et al.* an "afferent inhibition."

There are several significant lines of evidence in favor of this hypothesis. Mitchison & Dube (34) showed, first, that immune lymph nodes adoptively transferred to enhanced mice would block the typical response to a tumor homograft; the tumor failed to give enhanced growth. Second, they showed that the lymph nodes of mice enhanced and homografted were inactive; they could not adoptively transfer an immune response. These results indicate that it is lack of cellular immunity that causes enhancement, rather than any protection of the graft from this immunity. In the phraseology of Billingham *et al.*, the blockage is afferent and not efferent. Further evidence is provided by the demonstration by Snell (70) that addition to the tumor inoculum of normal lymphoid tissue of the same genotype as the tumor will counteract the enhancing treatment and prevent progressive tumor growth. The added lymphocytes may be presumed to reach the regional lymph nodes via the

lymphatic drainage system, despite the presence of humoral antibodies directed against them, and there to establish an immune response of the cellular type. The role of lymphoid cells as antigens in this type of immunity will be discussed further below.

Also significant is the demonstration by Bonfiglio *et al.* (71) that an enhancing treatment resulted in a lowered cellular infiltration of bone homografts in dogs. Demonstrable humoral antibodies were present. This could be interpreted in several ways, but in the context of the afferent blockage hypothesis might be taken to mean that the regional lymph nodes did not develop a cellular immunity and hence were unable to dispatch immune cells to the region of the graft.

The interesting observation by Green & Wilson (51) that the spleen does not enlarge in homografted enhanced rats as it does in homografted rats which have not been enhanced is at least not incompatible with the walling off hypothesis.

The "walling off" concept of the enhancing effect finds excellent precedents in classical immunology. Cannon (72), in a review of the functional significance of agglutinins and precipitins, finds extensive evidence for a localizing action of antibody on bacterial infections. Kahn (73), in ingenious experiments using diphtheria toxin as the antigen, has also demonstrated a remarkable degree of localization in immune animals. The immunity in this case, however, was not passively transferable and was presumed to be due to antibodies with an affinity for various tissues, particularly skin. The failure of passive transfer sets these antibodies apart from those studied by Kaliss in connection with the enhancing effect, but it may be asked if skin-localizing antibodies might also play a role in enhancement.

A modification of tumors in enhanced animals such that they grow more actively on subsequent homografting has been noted, and invoked as an explanation of the enhancing effect (68, 74, 75). Quite possibly this is a part of the process, in some cases at least. Part of this effect, however, may be due to the fact that the blood and infiltered lymphocytes of tissue taken from a homograft are those of the host and not the donor strain. The possible significance of this is indicated in the discussion in the next section.

Lymphoid Cells as Antigen in Graft Immunity

It may be presumed that for development in a grafted animal of an immune response, either of the cellular or the humoral type, the antigens of the graft must be brought into intimate association with the cells of the appropriate lymphoid organs. There are several ways in which this may happen. First, cells capable of an immune response may migrate to the graft. The presence in submaxillary gland homografts of mature and immature plasma cells, as noted by Darcy (24), suggests that this does occur. However, the draining lymph nodes of animals with homografts appear to develop an immune response before there are significant numbers of plasma cells in the graft, so if these cells are important, it is likely to be in the later phases of

the process. Second, it might be supposed that there are soluble isoantigens that pass in solution to lymph nodes or spleen. Arguing against this supposition is the insolubility of the *H-2* antigen in mice (44). Also Prehn & Main (76) found that blood enclosed in diffusion chambers in the peritoneal cavity did not immunize, while Merwin & Hill (77) found that small, nonvascularized grafts of Harderian gland or thyroid from prenatal or newborn mice did not immunize, though capable of responding to immunity otherwise induced. Third, in a vascularized tumor, or a tumor in proximity to lymph vessels, insoluble antigen or cellular debris may float to the appropriate organs in blood or lymph. There seems to be no evidence at present by means of which we can assess the possible significance of this alternative. Fourth, phagocytes may engulf antigen at the site of the graft and carry it to the lymph nodes or spleen. Descriptions of the cellular response at the graft site provide no clear evidence that this is part of the immunization process but phagocytosis of dying cells is a recognized process (78) and may play an important role in the establishment of graft immunity. Fifth, intact cells from the graft may pass directly to the lymph nodes via the lymphatic vessels. Several lines of evidence make it seem likely that this is an important factor in the development of the immune response. The evidence also suggests that donor lymphocytes in the graft play a particularly significant role.

The effectiveness of lymphoid tissues (thymus, spleen, lymph nodes, white blood cells) in evoking an immune response to subsequent grafts has long been known (79). Stoerk (80) has recently shown that liver, kidney, or spleen from cortisone-treated "donors," in whose tissues the concentration of lymphocytes may be presumed to have been reduced, showed a reduced capacity to immunize. Scothorne (81) likewise found that cortisone altered homografted skin so that it was less able to incite immunity, and Hardin & Werder (66) noted that the survival of skin homografts was prolonged by irradiation of the donor as well as of the host, a treatment that would selectively eliminate lymphoid cells. It is significant in this connection that lymphocytes are relatively abundant in skin (82, 83). The observation by Snell (70) that the addition of donor lymph nodes to the tumor inoculum will suppress the enhancing effect has already been mentioned. Added liver is without effect, in fact, may stimulate growth (unpublished data). Tumor plus lymph nodes in untreated mice grows less than tumor alone.

The ameboid motility of the leukocyte (84) and the abundant lymphatic capillaries in the skin together with the known rapidity of lymph flow (85, 86) provide the mechanical basis for rapid passage of lymphocytes from subcutaneous or intracutaneous grafts to nodes. Metastatic tumor cells may also pass to the nodes (87, 88), but available evidence does not suggest that this process is sufficiently rapid to account for the first appearance of the immune response.

Lymphoid tissue may also be particularly rich in isoantigens (or possess these antigens in particularly active form). Freeze-dried spleen is more effective in producing enhancement than any other normal tissue that has been tested (44). But liver is also effective in absorbing *H-2* antibodies (18).

Available evidence thus favors the view that donor lymphoid cells in the graft may pass rapidly to the regional lymph nodes and there incite an immune response. More critical evidence should be obtainable through the use of cells appropriately tagged. Other methods of antigen transfer probably also play an important role.

Some Factors Affecting the Type of Response

It will be helpful at this point to summarize briefly the facts so far presented. The inciting agents in the homograft reaction are isoantigens present in the graft but foreign to the host. These isoantigens are gene-determined. While there is a demonstrably different isoantigen corresponding to each allele at each and every locus, and hence probably many dozens of different forms of these substances, it is reasonable to assume that the isoantigens produced by the alleles at one locus are closely related chemically, and perform essentially identical functions in the economy of the cell. Since in the mouse there are something in the order of 14 or 15 histocompatibility loci, we may suppose that histocompatibility isoantigens fall into 14 or 15 related groups. Referring again to the mouse, one of these families of isoantigens, that determined by the *H-2* locus, is unique in the predominant role that it plays in the homograft reaction. According to the best available evidence these *H-2* isoantigens are carbohydrate-protein complexes. They are present in the cell surface, quite possibly also in other membraneous structures of the cell. We can only guess about the isoantigens determined by other loci, but an assumption that they are also present in cellular membranes, and that some at least of them are partly lipide in composition, seems plausible.

The immune reactions induced by these isoantigens (most of the reactions studied in detail have been due to the *H-2* antigen) may be of two kinds: (*a*) a humoral response demonstrable, among other ways, by the production of red cell agglutination; (*b*) a response localized within cells of the lymphoid tissues.

The cellular factors play a predominant role in the destruction of homografts. The humoral factor may produce diverse effects according to the nature of the target tissue. The growth of some tumor grafts is inhibited, the growth of others is enhanced, while still others seem not to respond at all. There is virtually no evidence indicating any effect of the humoral factor on homografts of normal tissue, although in some instances it may be that enhanced growth is induced.

The diverse effects produced by circulating antibody are one cause of the complexity of the phenomena associated with homografting. Another cause is the variation in the degree to which the two types of response are evoked by different methods of preparation and administration of the donor tissue or tissue extract. It seems likely that individuals effectively exposed in almost any manner to isoantigens will develop both cellular and humoral immunity in some degree, but the "balance" between these two may vary greatly according to the circumstances. The response of the graft will depend both on this balance, and on the nature of the graft itself.

We now turn to consideration of the various factors which may affect the type of immune response.

Living versus dead tissue.—The evidence is now rather conclusive that, with certain interesting exceptions, living tissue homografts or injections give rise to both the humoral and the cellular responses, whereas injections of nonliving tissue give rise only (or mostly) to the humoral. That living grafts produce both is shown clearly by the work of Mitchison (33, 34) discussed earlier. Evidence for the production of humoral antibody in animals receiving freeze-dried tissue or tissue extracts has already been cited. That cellular immunity cannot be present in these animals is indicated by the occurrence of the enhancing phenomenon, since the addition of the cellular factor to enhanced animals is all that is necessary to prevent the growth of a tumor graft. More specifically, Mitchison (34) has shown that the lymph nodes of mice which have been injected with appropriate killed tissue (enhanced), and then homografted, will produce hemagglutinins in their new host but will not protect against a tumor. The cellular factor is lacking.

The contrast between living and dead tissue is manifest in the difference in the cellular response to living as contrasted with frozen homografts (89, 90). The latter show a greatly reduced infiltration with leukocytes, the concentration of invading cells being comparable to that occurring in autografts.

A very important exception to the usual behavior of killed tissue has recently been reported by Billingham, Brent & Medawar (91). These authors have produced immunity to skin homografts in mice by means of cells disintegrated by ultrasonic irradiation. The active agent is relatively unstable but has been extracted in a nucleoprotein fraction. Kandutsch's studies (44) cited above cast some doubt on the conclusion of these authors that the antigens responsible for skin transplantation immunity are deoxyribonucleoproteins, since mucoproteins may well have been present as contaminants as they were in Kandutsch's carefully purified nucleoprotein fractions. If this be the case, it is unnecessary to assume, as Billingham *et al.* do, that histocompatibility genes produce antigens determining skin transplantation immunity which are entirely distinct chemically from other antigens produced by the same genes which act in other immune phenomena of transplantation. At least two alternatives are possible. First, the isoantigen in their extracts may have been a mucoprotein, with some other contaminant, or the deoxyribonucleoprotein itself, acting as an adjuvant. Second, as suggested to the writer by Dr. Kandutsch, their extract may have contained the typical mucoprotein isoantigen, but in unstable coupling with some other substance, perhaps a lipid, which altered the type, but not necessarily the specificity of the recipient's response. Whatever the ultimate outcome of this work may be, it remains a very interesting discovery. Some other exceptions to the general rule concerning living and dead tissue are cited below.

The time relations of the two responses.—In 1932, Dienes & Mallory (92) expressed the view that the tuberculin or cellular type of hypersensitiveness

represents the first stage of every immune response to injected protein. They found this type of hypersensitivity demonstrable in nontuberculous guinea pigs as early as the third day after sensitization by an intra-abdominal injection of egg white or horse serum. Mitchison's results with homograft immunity (33, 34) are in accord with an early appearance of the cellular factor. The humoral antibodies, and the capacity of the lymphoid organs to form them, persisted in his experiments long after the cellular immunity had dropped almost or quite to zero (Fig. 1A).

Whether humoral antibodies typically appear later than the cellular factor, or whether the two can appear at the same time is still not clear. Appearance of antibody three days after an initial stimulus has been reported (3, 93). The problem has an important bearing on the question of whether humoral antibodies are a necessary adjunct to the cellular factor in graft rejection.

An interesting consequence of the persistance of humoral antibodies after the cellular factor has disappeared is the occurrence of an enhanced response in the second of two tumor homografts given a month or more apart. It was shown by Kaliss (94) that the second of two transplants, if given soon after the first, showed the inhibition typical of a second-set response, but if given a month or more later showed enhanced growth. There may be great strain differences in this phenomenon [see for example (95)].

Dosage.—There is evidence that dosage has a pronounced effect on the type of response which appears following antigenic stimulation. The general rule seems to be that low dosage levels favor the development of cellular immunity.

Kaliss (96) studied the effect on the growth of subsequently implanted tumor of pretreating mice with widely varying doses of freeze-dried tissue. In the tumor-host combinations selected for this study, untreated controls gave about 50 per cent deaths. This made it possible to demonstrate easily either an enhancing or an inhibiting effect of the pretreatment. In seven different experiments, the groups receiving 5 or 50 mg. per mouse, showed, as expected, a higher proportion of mice dying. A dose of 0.005 mg., on the other hand, consistently reduced the number of deaths, while the effect of 0.5 and 0.05 mg. was variable. In the three experiments in which it was used, a dose as low as 0.0005 mg. per mouse appeared to produce some protection. Even at the level 0.005 mg., since the isoantigens can compose only a small fraction of whole freeze-dried tissue, an immunologic stimulation by very minute amounts of isoantigen is indicated. Since the small doses reduced rather than increased the growth of the tumor transplant, the presumption is that the response was of the cellular type. No tests were made for hemagglutinins, but it would be surprising to find them present in measurable quantity. Possibly this is a clue as to whether or not they play an accessory role in graft destruction.

Uhr, Salvin & Pappenheimer (97) have shown that delayed or cellular hypersensitivity is consistently induced in guinea pigs by the intradermal

injection of 1 or 2 μg. of an appropriate antigen-antibody complex. Maximal sensitivity develops at least two or three weeks before detectable circulating antibody is formed. Chase (98) found that five or six daily intracutaneous injections of picryl chloride gave rise to the delayed type of sensitivity, while with longer series of injections humoral antibody was formed in increasing amounts.

An increase, both relative and absolute, in the humoral response with continuing stimulation is suggested by Darcy's (24) observations on the histology of grafts, some features of which have already been discussed. In counts of cells in a given area of section of 8-day-old grafts, Darcy found 80 mature and immature plasma cells to 312 lymphocytes in first-set grafts, whereas the numbers in second-set grafts were 160 and 193, respectively.

An interesting question is what happens to cellular immunity when further stimulation, leading to intense humoral antibody formation, is applied. Chase (98) noted persistence of delayed sensitivity to picryl chloride in guinea pigs after continued injections had led to anaphylactic sensitivity. However, Mitchison's studies (33, 34) indicate that, at least in certain cases in mice, the cellular response may decrease. This is seen by comparing his results obtained following a second tumor transplant as contrasted with a first. While the humoral antibody response increased the second time, the cellular response went down (Fig. 1B as compared with A). A form of enhancement noted in both tumor and normal tissue grafting (99, 100), following repeated transplants can perhaps be explained in this fashion. It should be noted that Gorer (3) doubts the significance of Mitchison's results on this particular point.

The effect of adjuvant.—While there is no published evidence that the homograft reaction can be altered by the addition of adjuvants to the immunizing tissue or tissue extracts, the effect of adjuvants on immunizing processes is too well-known to require elaboration. While adjuvants increase the immune response in general, there is in many situations a tendency to favor the delayed type of hypersensitivity (101, 102). We have already suggested that the results of Billingham, Brent & Medawar (91) with nuclear extracts may be due to an adjuvant effect.

Route of injection.—The cellular type of immune response is favored by the intradermal route of injection (103). In keeping with this, Medawar (104) found that leukocytes were eighteen times more effective in eliciting immunity to a subsequent skin homograft in rabbits when given by the intradermal route than when given intravenously. The injection of whole blood (105) or of suspensions of normal cells (unpublished data of the author) by the intra-abdominal route is also an effective method of immunization against grafts, although where tumors provide the test system immunity due to circulating antibody may make interpretation of these results uncertain. There is some indication that the injection of living cells by the intravenous route may favor the enhancing response. Snell *et al.* (106) have reported enhancement of tumor homografts by the intravenous injection of

whole blood, and Billingham & Sparrow (65) who, alone among the investigators who have successfully enhanced normal tissue, have done so with living cells, also employed this route. The same preparations given intraperitoneally were without effect, and inhibited if given intradermally. Parabiosis, which has been shown to lead to successful skin grafting in certain instances (107), may involve the same principle since it leads to a reciprocal exchange of blood. It is noteworthy also that the intravenous route has been regularly employed where a tolerant condition has been induced in irradiated mice by the injection of bone marrow, [see, for example (108, 109)]. This, however, may represent a special situation more akin to actively acquired tolerance.

Differences in the host strain.—While there has been one study (110) showing strain differences in antibody production in mice, there is little published evidence clearly indicating strain differences in the homograft reaction. In the author's experience, however, certain strains, e.g., C57BL/6Ks and C57BR/a, are much more susceptible to the enhancing effect than others. A persistence of ability to transfer cellular immunity noted by Billingham *et al.* (36) up to 30 days after a graft of skin had been made from A donors to CBA hosts, as compared with the ten days reported by Mitchison (33), may well be due to the use of different strains. The C57BR/a's on which most of Mitchison's data are based are easily enhanced, which is consistent with the development of a relatively transitory cellular response. Species differences may be expected to be at least as important as strain differences.

Differences in the histocompatibility locus.—Different histocompatibility loci determine different isoantigens, and it may well be that they will show interesting differences in the type of immune response which they evoke. This is an untouched field, but one which should be susceptible to investigation by the use of coisogenic strains (111) differing from one another at one histocompatibility locus only.

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USE OF TISSUE CULTURES IN VIRUS RESEARCH[1,2]

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During the last half decade, newer knowledge of mammalian cell culture has both resulted from and given rise to increasing practical and theoretical application to virus research. With development of true cell culture from earlier tissue culture technics, it has become possible to analyze for animal cells the obligate dependency of virus on host cell in manner similar to that applied with microbial cells. The accompanying study of such specific properties of animal cells as capacity to absorb virus, propagate virus, and respond to virus infection in a manner other than destruction, has advanced the science of cell physiology as well as that of virology.

This review is intended to be (*a*) a guide to uses of cell culture in virus research, and (*b*) a source of access by the reader to the complete literature of each method or system discussed. References cited, amounting to about half of those published from 1950 to 1956 were selected, not for critical review of results, but to provide a variety of material illustrating approaches or applications of cell culture. Since these approaches can and have been used to study cells apart from viruses, the content of this review has been organized to attract broader interest than that of the strict virologist. In any case, even he cannot employ cell culture efficiently without being something of a cell culturist.

The literature surveyed continues from the last specific reviews of the application of tissue culture to virus research by Robbins & Enders (1) and Gey & Bang (2). Some aspects of the subject have since been considered by Sanders *et al.* (3), Heys (4), and Weller (5). The general sources of recent literature on tissue and cell culture are indicated by pertinent citations in the text.

Cell Culture Techniques

Dispersal of cells.—One technique, perhaps more than others, has been responsible for the rapid advances in virological cell culture in recent years, that for dispersal into suspension of cells from tissues or cultures. Revival by Scherer, Syverton & Gey (6) of the Rous-Jones application of trypsin digestion to dispersal of cells and its application to strain HeLa cells, made pos-

[1] The survey of the literature pertaining to this review was concluded in December, 1956.

[2] The following abbreviations are used: DNA (deoxyribonucleic acid); DPN (diphosphopyridine nucleotide); FAD (flavin-adenine-dinucleotide); FMN (flavin mononucleotide); TPN (triphosphopyridine nucleotide).

[3] The experimental studies from the authors' laboratories were aided by Grants from the National Foundation for Infantile Paralysis, Inc., The National Cancer Institute, and The American Cancer Society.

sible a standardized routine propagation of epithelial as well as fibroblastic cells in continuous culture. Use of trypsin by Frisch & Jentoft (7) for the preparation of monkey testicular primary cultures foreshadowed widespread use in the preparation of monkey kidney cultures. Later refinements included (*a*) use of Moscona's (8) calcium-magnesium-free salt solution as diluent to reduce aggregation of dispersed cells; (*b*) repeated extraction of small fragments of monkey kidney tissue agitated in replenished trypsin solution in a Waring blendor [Youngner, (9)]; (*c*) systematic investigation by Rappaport (10) of trypsinization procedure resulting in an apparatus for the automatic extraction of tissue; and (*d*) the simplified procedure of Bodian (11) for overnight extraction of magnetically stirred monkey kidney suspensions in a refrigerator. The separation of cells by the chelating action of sodium versenate, introduced by Zwilling (12), has been useful for the disruption of cell culture monolayers, but not for the preparation of cell suspensions from tissue. Pancreatin, recommended by Hanks (13) as a superior declumping agent, has not been as widely used as has trypsin.

Growth media.—Stewart & Kirk (14) have summarized earlier physiological research with serum tissue extract growth media for cell culture. Advances made by or for virological research have proceeded in two directions: (*a*) toward the use of cheaper bacteriological-type media for mass growth of cells, and (*b*) toward synthesis of the long sought, fully defined growth media. Economy of serum requirement for culture growth resulted from introduction by Robertson, Brunner & Syverton (15) of yeast extract for culture of HeLa cells. This supplement also aids derivation and routine management of established cell strains of other types (16). In similar fashion, lactalbumin hydrolysate [Melnick & Riordan (17)] has been an inexpensive and effective nutrient for monkey kidney (18) and other cells. Mayyasi & Schuurmans (19) obtained superior growth of strain L cells by reinforcement of yeast extract with proteose peptone #3, among a wide variety of bacteriological supplements tested, in comparison with egg-ultrafiltrate-solution 199 reference medium. In combination with horse serum, the supplements gave good growth of strain Maben and other cells. For growth of strain L cells, filterable chick embryo extract was prepared by ultracentrifugation and treatment with hyaluronidase by Bryant, Earle & Peppers (20). Earlier studies showed that filtered extract could be replaced with an ultrafiltrate [Sanford *et al.* (21)]; ultimately a, cheaper and more convenient stimulant was provided in the form of whole egg ultrafiltrate [Earle (22)]. Horse or calf serum or other animal fluid such as bovine allantoic and amniotic fluid (23), were used to extend or replace expensive human serum for cultivation or maintenance of human cells. Earle *et al.* (24) were able to adapt HeLa cells to continuous growth in horse serum by progressive replacement of the human serum ordinarily employed. Many laboratories now carry substrains of cells adapted to growth in heterologous serum medium. Development of more defined media for cultivation of strain L mouse cells has proceeded rapidly, at present culminating in the albumin-peptone-supplemented solu-

tion of Waymouth (25) and the improved protein-free medium of Evans *et al.* (26). Continuing work by Healy *et al.* (27) yielded medium 858, containing lipid sources, coenzymes, nucleotides, and deoxyribosides in addition to amino acids and vitamins. These efforts, aimed at producing defined media for continuous growth of cells, were paralleled by Eagle's definition of the vitamin, amino acid, and other nutritional requirements of strain L and HeLa cells. Although the defined medium (28) was not complete without serum, its simple formula made it ideal for growth and maintenance of cells for virus studies.

Maintenance media.—Media for maintenance of cellular metabolism without stimulation of multiplication required for cell cultures used in detection and quantitation of virus, have been steadily improved. The ideal medium for these purposes should, without renewal, maintain cellular metabolism and preserve cellular morphology unaltered for at least 10 to 14 days of incubation at 37°C. It should be economical, reproducible, and stable on storage. Suitability for quantitative studies has made the strain L cell a predominant subject of nutritional research; unfortunately for virological needs, this cell is less useful than others. Differing requirements of the many cell strains in common use have resulted in considerable variation between laboratories in details of formulas for maintenance media. Although no one medium can be recommended for general use, it is helpful to consider classes of useful materials which have been incorporated in maintenance media. Commonly, a defined medium is employed alone or as basal solution in place of simple, balanced salt solution. These defined media also have largely replaced earlier employed serum ultrafiltrates. Medium 199 of Morgan, Morton & Parker (29) has been employed extensively. It was used by Wood *et al.* (30) with roller-tube cultures of monkey testis maintained for detection or propagation of polioviruses. Farrell *et al.* (31) used medium 199 to maintain monkey kidney tissue for large-scale production of polioviruses for use in vaccine; this medium has been used routinely for production of Salk vaccine (32). Later modifications have been described (33). Scherer's (34) solution (MS) as recommended by Syverton, Scherer & Ellwood (35), with or without yeast extract, has been commonly used in maintenance media for HeLa and other cells. Other defined solutions [Waymouth (36); Morgan *et al.* (37)] may be equally useful for maintenance of particular cells. Rappaport (38) has evaluated and recommended a simple buffered synthetic solution containing cysteine, *iso*-leucine, ribose, and glucose for outgrowth of monkey kidney cells and propagation of poliovirus in the absence of serum. Various organic supplements have been employed to reduce the small content of heterologous serum commonly employed in cellular maintenance media. Lactalbumin hydrolysate (17) is widely used for both establishment and maintenance of monkey kidney cultures (18). Enders (39) employed bovine amniotic fluid as a serum substitute and buffer for propagation of several viruses in primary cultures of human tissues. Bazeley, Rotundo & Buscheck (40) measured the capacity of plasma derivatives to

reinforce medium 199 in place of horse serum for establishment of primary cultures of trypsinized monkey kidney cells in bottles or roller tubes. Cohn fractions IV and V of human plasma enhanced culture growth at least as well as horse serum; increased yields of Type 1 poliovirus were given by cultures so propagated and maintained in medium 199 alone, possibly by reason of greater cell populations and utilization of damaged and destroyed cells for pabulum. The same bovine plasma fractions were somewhat less active. Yeast extract medium, originally described (15) for maintenance of HeLa cells in colorimetric assays of poliovirus, has been useful with added buffer for maintenance of stationary tube cultures. Morgan, Campbell & Morton (41) have tested varying levels of a number of natural materials as supplements to defined medium M150 for maintenance of primary cultures of explanted chick heart. Liver extract, horse serum, and yeast extract were active in that order. Ginsberg, Gold & Jordan (42) showed that addition of tryptose phosphate broth to Scherer's maintenance solution containing 7.5 per cent chicken serum, among several bacteriological broths, not only permitted establishment in culture of mechanically dispersed HeLa cells but maintained the integrity and morphology of cultures for seven to ten days without feeding. Considerable multiplication of HeLa cells indicated that the tryptose phosphate medium was not a true maintenance medium. It was not surprising that higher titers of adenoviruses and Type 1 poliovirus, and increased yields of adenovirus, were obtained with HeLa cultures so maintained. Buffering of media, a problem in virus studies, has been improved in some cases by use of tris(hydroxymethyl) aminomethane (commonly called "tris") to replace or reinforce bicarbonate and phosphate. Swim & Parker (43) found that primary cultures of human tissues tolerated tris well; HeLa and L cells were somewhat more sensitive but proliferated in spite of morphological changes. Respiration of HeLa cells was found by Gifford, Robertson & Syverton (44) to continue undiminished for 78 hr. in the presence of 0.01 *M* tris or 0.02 *M* sodium hydrogen phosphite.

Cell culture methodology.—Culture techniques now used in virus laboratories are directed toward three ends: (*a*) development of strains of cells in continuous culture; (*b*) maintenance of stock cell cultures; and (*c*) mass preparation of cell cultures for growth or assay of virus. Procedures employed for particular cells vary with the laboratory and purpose of culture; standard methods have been reviewed recently by Melnick (45). Since virus research mainly demands large numbers of healthy replicate cultures, procedures for counting and dispensing of cells must be chosen with regard for economy and time consumed in operation as well as exactness. Precise methods for counting cells or evaluating culture growth, such as enumeration of cell nuclei [Sanford *et al.* (46)], assay of deoxyribonucleic acid phosphorus [Healy *et al.* (47)], or protein [Oyama & Eagle (48)], are more commonly employed in studies of cellular nutrition or physiology. Direct counting of trypsinized cells (35) or estimation of packed cell volume (49), although less exact, is more convenient in preparation of replicate cell cultures for virus

studies. The prototypical techniques of Evans *et al.* (50) and Parker *et al.* (51) for mass dispensing of replicate cultures likewise have been simplified by virologists. Cultures used for detection, growth, or assay of viruses commonly are dispensed by serological pipette or Cornwall pipetting unit (9, 35) from cell suspensions dispersed with trypsin or versene (18, 52) and standardized by count of nuclei or whole cells or by optical density (9). Cells are propagated in rubber-stoppered [Parker, Morgan & Morton (53)] tubes for virus titrations, in large flasks or bottles (54) for production of of stock or vaccine virus, or in Petri dishes (55) or prescription bottles (56) for virus plaque counts. Other types of biological dishes (57) also can be used for cell cultures. A variety of culture forms have been developed for physiological investigation, including cell cultures in sponge matrix (58), on glass wool (59), under cellophane (60), and on collagen (61). These techniques have not been widely employed in virus work, but may have value for obtaining growth of fastidious cells or for observation of cell morphology.

Production of source cells for mass preparation of cultures remains expensive and time-consuming. While cell strains in continuous culture, particularly clonal strains (16, 62), are excellent sources of morphologically similar or genetically pure cells, maintenance of stock cultures may be too costly for the small laboratory without continuous demand for cultures. For such laboratories, cells dispersed with trypsin from such tissues as kidney and amnion provide ready means for intermittent production of numerous cultures. Primary cultures have serious disadvantages because of (*a*) the mixed nature of cell populations, and (*b*) the common presence of contaminating viruses originating from source animals. It should be noted that the continuous stable culture is not necessarily exempt from the hazard of microbial contamination (63) as was warned by Gey *et al.* (64).

Derivation of new cell strains depends on many still undefined factors. Procedures reported successful usually involve primary culture of surgical tissue specimens or biopsies in rolled or stationary plasma-clot cultures, harvest of cells growing in areas of clot liquefaction (65) or growing out beyond the original clot [Hull (66)] or dispersed by trypsin digestion of the coagulum (67), and direct [Shannon & Earle (68)] or subsequent subculture on glass. Usually cells can be considered established as a stable strain when they have been maintained in continuous culture for a year. Then, or earlier, clonally derived substrains can be obtained by plating dispersed cells with or without aid of a feeder layer [Puck & Marcus (69)].

Apparatus.—Specialized glassware has contributed materially to the precision and convenience of cell cultural virus research. Elaboration by Earle and co-workers of quantitative techniques for culture of strain L cells or cells from soft tissues produced (*a*) the T series of plane-floor flasks (70) for culture and quantitative recovery of cells; (*b*) sieving apparatus for preparation of mechanically dispersed cell suspensions (71); (*c*) apparatus for quantitative dispensing of replicate cultures (50); and (*d*) pipettes for quantitative enumeration of cell nuclei (46). All of these components are

now commercially available. A simpler version of Earle's apparatus was designed by Syverton, Scherer & Ellwood (35) for dispensing of HeLa and other epithelial cells. Tubes closed by rubber-lined screw caps have come into general use as culture vessels, but are unsuited to large-scale use because of expense and the manual labor of screwing on caps. Barski (72) has used cups formed in plastic sheet for cell culture virus assays, and Rightsel *et al.* (73) have adapted a commercially available vinyl artist's cup panel. The panels, simultaneously sterilized and cleaned with alcohol, are sufficiently inexpensive to discard after use. Sealed with cellophane tape or mineral oil, the panel cups are used for colorimetric virus assays; the transparent plastic permits microscopic observation of cultures if desired. The plane-floor Petri dishes of Warren *et al.* (74), which facilitate application of phase microscopy to observation of cellular morphology, have been made available commercially. These should be useful for visualizing cytopathology. Harris & Barclay (75) have contributed a slide cell permitting concomitant observation of cell cultures and electrometric measurement of oxygen consumption. Lwoff *et al.* (76) adapted a micromanipulator to study of virus propagation by isolated cells, and were able to relate stages of virus release to cytopathology.

Storage and transportation of cells.—Preservation of cells has made it possible to widen and enlarge the range of their usage. Cost of cell cultures irregularly required in quantity can be reduced by suitable preservation of source tissues, cell suspensions, or established stock cultures. Continuously cultivated stable or clonal strains may be maintained in fluid medium at 31° to 32°C., 4° to 5°C. [Swim & Parker (77); McAllister & Coriell (78)], or in agar medium at room temperature [Puck, Marcus & Cieciura (79)]. Frisch & Jentoft (7), Melnick (18), and others have found that suspensions of tissue fragments or dispersed tissue cells in suitable medium can be kept refrigerated for some days for later culture. Scherer & Hoogasian (80) evaluated conditions required for preservation of strain L mouse and HeLa human cells by freezing at −60° to −70°C.; cells suspended in suitable concentrations of glycerol or serum and thawed quickly were recovered after six months. In this laboratory, cells of these and some other strains have been recovered readily and repeatedly after storage frozen in serum diluted with quantities of glycerol suited to particular cells. The possibility that frozen storage produces changes in cultivated cells similar to those observed by Morgan *et al.* (81) for mouse ascites tumor cells has not been excluded. As shown by Scherer, Syverton & Gey (6), Melnick *et al.* (82), Lipton & Steigman (83), and Scherer & Brown (84), capacity of cultivated cells to withstand relatively unfavorable environments makes possible commercial shipments over long distances. Poliomyelitis and other virus research has been facilitated by shipment of cell cultures from central preparatory laboratories or commercial sources. For the small laboratory, purchase of prepared cell cultures eliminates cost of glassware cleaning and source animals or maintained stock cultures.

Culture techniques for virus study.—For specific application in virus research, animals cells are used in two forms: (*a*) as sheets of contiguous cells about one cell thick (monolayers), or (*b*) as suspensions of dispersed cells in fluid medium. Essentially all virus research techniques are directed toward a few ends: qualitative isolation or detection of infectious virus or both, production of virus in quantity, direct or indirect visualization of effects of presence of one or more virus particles, and isolation of the progeny of particular virus particles. Major techniques available for study of viruses are typified by those developed in recent years for poliomyelitis research; these are increasingly being applied with other viruses.

For *in vitro* cultivation of viruses, cells grown out from tissue fragments embedded in plasma coagulum (that is, true tissue cultures) replaced the tissue-fragment suspensions of the Maitland-type (85) earlier employed. As Morann & Melnick (86) showed with monkey kidney epithelium, equally satisfactory bottle or tube cultures could be prepared without plasma. Removal of original explants before virus inoculation of remaining cellular outgrowth produced the forerunner of the true cell culture. Cultures propagated from cells obtained by tryptic digestion of cell colonies in continuous culture (6) or tissue fragments (9, 55) became the major type for virus research. The problem of incubator space for the large number of cultures required for virus titrations was eased by satisfactory replacement of roled (87) with stationary cultures (88).

Evolution of the glass-borne cell culture facilitated definition of cultural conditions affecting virus production *in vitro* [Scherer (89); Youngner (90)]. Adaptation of tissue and cell cultures to virus research in this country was paralleled or succeeded by similar developments abroad [Bazeley & Thayer (91); Kret (92); Sheffield & Smith (93)].

Use of trypsin to disperse cells resulted in a second contribution to virological cell culture, by allowing exploitation of the effect of virus infection on cell metabolism noted by Enders, Weller & Robbins (94). Cells dispersed in fluid suspension were rapidly destroyed after exposure to free virus, or survived and continued to metabolize after exposure to neutralized virus. With suitable media, the outcome for cells derived either from tissue or stable strains was revealed visually by change in color of phenol red pH indicator in the medium; on the basis of this phenomenon, colorimetric assays for poliomyelitis virus and antibody were designed by Robertson, Brunner & Syverton (15), Salk, Youngner & Ward (33), and Lipton & Steigman (95). Conjoined use of (*a*) the colorimetric principle, (*b*) disposable plastic panels, (*c*) mineral oil for sealing panel cups, and calf serum-lactalbumin-hydrolysate medium for maintenance of monkey kidney cells dispersed from primary culture with versene, together with use of mechanical pipettes for dispensing solutions, formed an economical cell culture system described by Melnick & Opton (96) for large-scale screening and qualitative titration of poliomyelitis virus antibody.

Techniques described by Dulbecco & Vogt (97) for quantitative assay of

animal virus particles by plaque count initiated quantitative studies of virus-cell relations. For plaque assays, monolayer cultures were grown from trypsin- or versene-dispersed cells in Petri dishes; established monolayers were washed free of serum antibody, inoculated with virus, and overlaid with washed agar medium. Plaque technique has since been simplified by replacement of Petri dishes with flat-sided bottles to eliminate need for gassed humified incubators, and by use of unwashed agar. Cooper's (98) method of seeding virus particles into heavy concentrations of cells suspended in melted agar represents an efficient and convenient, but expensive, system of plaque assay more versatile than the monolayer system. Methyl cellulose gel has been described by Hotchin (99) as a suitable nonfluorescent substitute for agar in identification of plaques with fluorescent antibody.

Use of mechanically, enzymatically, or chemically dispersed cells, rather than suspended tissue fragments (100) greatly increased precision of kinetic studies of virus growth by permitting control of cellular as well as virus populations. As described by Pereira (101) and Rubin *et al.* (102), chick embryos can be convenient sources of cell suspensions and monolayers. The more homogeneous cell populations represented by stable or clonal strains of cells in continuous culture make these superior subjects for kinetic study. Special techniques are necessary to maintain epithelial cells such as those of strain HeLa in dispersed suspension. Girardi *et al.* (103) reported that strain HeLa cells could be kept dispersed for a reasonable period in silicone-coated flasks by agitation with magnetic stirring apparatus. Viable cells are differentiated by capacity to absorb neutral red or reject trypan blue. Neutral red is also used commonly to add contrast for detection of virus plaques in monolayer cell cultures; dye solution can be applied directly to the agar overlay or allowed to diffuse downward from a secondary stain-agar overlay.

Gifford, Robertson & Syverton (104) showed that continuous measurement of oxygen consumption by trypsin-dispersed HeLa cells dispensed into conventional Warburg flasks permitted simultaneous assessment of the physiological state of host cells, and their response to infecting virus. The method was found useful for testing potential virus inhibitors, and for investigation of the physiology of virus infection (105).

Properties of Applicable Cells

Available cells.—Tissue by primary culture provides a ready source of cells useful for simple detection or production of virus, or for preliminary assessment of viral susceptibility. For the latter purpose, if only small amounts of tissue are available, outgrowth may be used from explant cultures prepared in flasks or tubes by the older techniques utilized so widely in poliomyelitis research (106, 107, 108). Cells in larger numbers can be simply harvested from soft embryonic (41) or tumor (109) tissue by passage through stainless steel mesh inserted in a syringe, and subsequent sieving through finer mesh, glass wool, or porous sintered glass. Embryonic or adult kidney tissue constitutes a major source of epithelial cells obtainable by ex-

traction with trypsin. By suitable methods, kidney cells of various monkeys and other animals appear equally cultivable [von Magnus *et al.* (110); Millian & Robbins (111)]. Human tissue for direct culture usually is available as surgical or obstetrical material; particular attention has been paid to cells obtained from human amnion [Zitcer, Fogh & Dunnebacke (112); Weinstein *et al.* (113)]; amnion and chorion [Lahelle (114)]; and fallopian tube tissue [Pizarro & Pérez-Rebelo (115)]. Cultivation of Eastern equine encephalomyelitis virus in teleost embryos by Sanders & Soret (116) suggests that cultural procedures used with mammalian cells may be applicable in virus studies with cells of lower animals.

The clonally derived, genetically homogeneous cell strain in stable continuous culture represents the ideal *in vitro* host for virus study. Plating technique of Puck *et al.* (79) has readily yielded clonal substrains from a variety of parent stable cell lines (16, 62). The list of established cell strains, potential or actual sources of a wide variety of genetically pure animal cell types, has lengthened rapidly in recent years: description here will be limited to those cultivable on glass from dispersed inocula, propagated by described procedures, characterized with respect to biological behavior and viral susceptibility, and generally available.

The pure mammalian cell strain designated "L" was clonally derived by Sanford, Earle & Likely (117) from an established fibroblast strain originally explanted by Earle (118) from subcutaneous tissue of a C3H mouse. While exposed to 20-methyl-cholanthrene in culture, the original strain underwent neoplastic transformation (119). Adaptation of L cells to growth on glass by Shannon & Earle (68) provided the prototype of ideal cell populations for virus studies. Scherer (120) demonstrated cellular agglutination and antibody-induced, agglutination-inhibition by exposure of L cells to vaccinia virus. Mumps and influenza A viruses were not similarly active, and vaccinia virus did not appear to multiply in L cultures. L cells were susceptible to infection by pseudorabies and herpes simplex viruses [Scherer (34)], reflected by rounding, detachment of cells from glass and appearance in some cells of Type A intranuclear inclusion bodies. L cells responded to infection by the viruses of lymphocytic choriomeningitis and MM strain encephalomyocarditis by production of virus without overt evidence of cellular destruction (121). Strain HeLa cells originated as a stable strain explanted from an epidermoid cervical carcinoma by Gey (122) and established in continuous culture as cell colonies in plasma coagulum. A subline was transferred to the University of Minnesota in 1952, and there adapted to propagation in monolayer from cell suspensions dispersed with trypsin; the cells were shown susceptible to destructive cytopathogenic effect of the three types of poliovirus [Scherer, Syverton & Gey (6)]. Easily propagated by routine procedures, the strain was developed as a tool for laboratory diagnosis of acute poliomyelitis (123) and for large-scale isolation and typing of field strains of poliovirus [Syverton, Scherer & Ellwood (35)]. The cells supported multiplication of herpes simplex, pseudorabies, and vaccinia viruses (124), responding with

formation of inclusion bodies and cytopathogenic focal destruction. The arthropod-borne viruses also multiplied in HeLa cells; Eastern equine encephalomyelitis virus was rapidly destructive, but Western equine encephalomyelitis, West Nile, St. Louis encephalomyelitis, and Japanese B encephalomyelitis viruses were irregularly cytopathogenic [Scherer & Syverton (125)]. HeLa cells were refractory to at least some strains of lymphocytic choriomeningitis, encephalomyocarditis, and mouse encephalomyelitis viruses (126). Murphy, Hubbell & Yager (127) were able to propagate Venezuelan equine encephalomyelitis virus in HeLa cells. Cytolytic effects on HeLa cells typical of poliomyelitis infection were produced by Coxsackie viruses B 1 to 4 and A 14 [Crowell & Syverton (128)]. Habel, Gregg & McBride (129) found a HeLa subline adapted to growth in horse serum as susceptible to Types 1–3 polioviruses as control monkey kidney cultures. As summarized by Rowe, Hartley & Huebner (130), lately HeLa cells have been employed in characterization of the new adenovirus group; most members of the group are cytopathogenic for HeLa. For isolation and typing of enteric cytopathogenic human orphan (ECHO) viruses, monkey kidney cells are superior to HeLa (131); usually these viruses do not propagate readily in HeLa cells without adaptation. In 1954, Chang (132) reported successful serial subcultivation of epithelial-like cells from normal human conjunctiva, liver, kidney and appendix; tissue explants were initially established in plasma clot culture and cells dispersed with trypsin for transfer. Nutrient quality and toxicity of individual homologous sera were shown to be important factors in successful establishment of the strains. Cells of these strains were propagated in medium containing human serum and chick embryo extract and maintained satisfactorily in horse serum-maintenance solution. Types 1–3 polioviruses multiplied in the conjunctival cells with specific cytopathogenic destruction; conjunctival, kidney, and appendiceal cells reflected about equal titers of Type 1 poliovirus. McAllister & Coriell (78) described standard procedures for enumeration of viable (neutral-red stained) trypsin-dispersed cells of the conjunctival and kidney strains, mass dispensing of magnetically stirred cell suspensions by automatic syringe and, finally, propagation in tubes or in small or large bottles. The cells multiplied about equally well in Chang's medium (132) with human serum or in solution 199 containing horse or calf serum; Eagle's medium (28) was somewhat less effective. Cells of both strains were readily adapted to growth in horse or calf serum medium, satisfactorily maintained or stored at 32°C. or 5°C. (conjunctival cells also at −70°C.), and were microbiologically sterile on injection into suitable animals. As in Chang's experience, some (although a smaller number of) human sera appeared toxic for the cells. Growth curves of Types 1–3 polioviruses propagated by the cells were similar in shape and yield to those obtained with first generation rhesus monkey kidney cells, and susceptibility to polioviruses was not altered by continuous cultivation of cells in horse serum medium [McAllister *et al.* (133)]. In propagating Chang's liver and conjunctival human cell strains, Syverton & McLaren (16) seldom experienced difficulty with toxic human sera by rou-

tine use of yeast extract medium. Clonal as well as parental liver lines were as susceptible to Types 1–3 polioviruses as cells of the conjunctival strain or cells of strain HeLa and cynomolgus monkey kidney. Eagle (134) established a new strain of human epithelial cells (KB) by directly planting on glass cells dispersed by trypsinization of minced epidermoid carcinoma tissue. The cells, propagated continuously in Eagle's defined medium supplemented with 10 per cent fresh human serum, after the second transfer maintained a growth rate higher than that of HeLa cells similarly cultivated in the same medium. KB cells in process of destruction replicated Type 1 poliovirus, Types 1, 3, and 4 adenoviruses, and the viruses of herpes simplex, vaccinia, lymphocytic choriomeningitis, and encephalomyocarditis; SV_1 adenovirus, mumps, and yellow fever viruses multiplied but were irregularly cytopathogenic; rabies virus was cytopathogenic only in primary passage; Theiler's GD VII mouse encephalomyelitis, A 9 Coxsackie, Type B influenza, and strain DC adenovirus neither multiplied nor were cytopathogenic [Eagle *et al.* (135)]. Transfer to glass of cellular sediment from a patient with metastatic pulmonary adenocarcinoma by Frisch *et al.* (136) yielded a strain of presumably malignant human epithelial cell (Maben). The cells were cultivated initially in 40 per cent pleural fluid diluted in medium 199 and subcultured by transfer of cells gently scraped from the parent growth. Later Maben cells were adapted to growth in 20 per cent normal human serum, 80 per cent medium 199, and dispensed for transfer by use of trypsin. At the time of reporting (more than two years after initial culture), Maben cells were resistant to low pH and sensitive to alkalinity and lipemic serum; they had been successfully preserved at −70°C. for at least two months. HeLa and Maben cells appeared equally sensitive and useful for isolation, titration and propagation of field, prototype and adapted strains of Types 1–3 polioviruses. Preliminary tests showed susceptibility to vaccinia and herpes virus, but not to influenza, mumps, lymphocytic choriomeningitis, Western equine encephalomyelitis, or psittacosis viruses. Moore, Sabachewsky & Toolan (137) derived four stable strains of human cells, designated *H.Ep.#1* (from epidermoid cervical carcinoma carried in irradiated and cortisonized rats), *H.Ep.#2* [from plasma-clot culture of pharyngeal epidermoid carcinoma explanted by Fjelde (138)]. *H.Ep.#3* (from lymph node invaded by metastasis from buccal epidermoid carcinoma), and *H.Emb.Rh.#1* (from embryonal rhabdomyosarcoma). All of these strains were first established in treated rats, kept by cultivation *in vitro* for months and, finally, readily regrown *in vivo* with resumption of original tumor morphology. Culture characteristics, morphology, reaction to heat and ultraviolet light, and confirmation of human origin were reported. Black, Reissig & Melnick (139) stated that Hep-2 (*H.Ep.#2*) was "easily grown in the absence of human serum," and that two strains of measles virus had been adapted to growth in these cells with typical cytopathogenic effect (multinucleate giant cells with intranuclear inclusions).

The experiences of Berman, Stulberg & Ruddle (140) in isolating the Detroit strains of human cells illustrate the common problem of determining

cellular origins. The Detroit-6 strain of epithelium-like cells arose in bone marrow culture after 51 days of incubation, as cellular plaques among proliferating fibroblasts. Prior to appearance of the plaques, cultures on glass of aspirated bone marrow suspensions showed usual sequential predominance of myeloid cells, histioid and monocytic cells, and, finally, proliferating fibroblastic elements. The epithelium-like cell colonies were transferred after mechanical detachment to subculture and successfully transferred thereafter by dispersal with trypsin. Morphologically more than culturally, Detroit cells resembled HeLa cells. Whether the cells originated from marrow metastasis of the carcinoma suffered by the donor patient, were derived from unknown normal epithelium sources, or represented a permanently altered form of another cell type, was not known. Detroit-6 cells, cultivated by procedure standard for HeLa, exhibited cytopathogenic effect and propagated Type 1 poliovirus, Conn. 5 strain of B 1 Coxsackie virus and herpes simplex virus; Types 2 and 3 poliovirus also were cytopathogenic but evidence for propagation was not reported; neither propagation nor cytopathogenicity was observed with influenza viruses [Stulberg, Berman & Ruddle (141)]. Interestingly, Detroit-6 cells exhibited a tendency toward aggregation with subsequent outgrowth of cell sheets from coalesced aggregates more characteristic of fibroblastic than epithelial cells. By similar methods, Berman & Stulberg (142) derived eight more epithelium-like cell strains: three (Detroit-6, -32, and -34) from cultures of sternal marrow taken from patients with primary or metastatic carcinoma; two (Detroit-52 and -98) from cultures of sternal marrow taken from patients without known malignant disease; two (Detroit-30A, -56A) from direct culture of carcinomatosis ascitic fluids; and one (Detroit-116P) from similar culture of lymphomatosis peritoneal fluid. The question of origin of these cell strains is additionally complicated by absence of gross morphological differences among the strains, their origin in all cases as epithelium-like plaques developing in parent cultures after successive progression through phases of myeloid, round cell, and fibroblast dominance, and the appearance of similar altered cells, whether or not the original culture source was associated with malignancy. Colonial elements could be subcultured simply by rapid passage of mixed cultures, since polygonal cells ultimately overgrew fibroblastic elements. All eight cell strains were reported (143) susceptible in both primary and secondary passage to cytopathogenic effect of adenoviruses 3 and 4, Coxsackie B 1, Eastern equine encephalomyelitis, herpes simplex, a strain of influenza A, lymphocytic choriomeningitis, Newcastle disease virus, Types 1–3 polioviruses, St. Louis encephalomyelitis and vaccinia viruses. Detroit-6, -98 and -116P cells supported multiplication of these viruses.

Jordan (52) employed autologous human serum for plasma-clot culture of human nasal mucosa taken during elective surgery. Secondary cell cultures were established by use of tryptic digestion. Two stable cell strains, DMB and DHov, were established by separation of areas of epithelial-like cells appearing among the predominantly fibroblastic elements of later sub-

cultures. Cells of these strains were dispersed with versene, and serially propagated in 40 per cent pooled human serum and 60 per cent Hanks' balanced salt solution. There was a tenfold increase in cell number after six days of incubation. DMB cells were maintained well by Ginsberg's medium (42), much better than DHov cells. DMB cells were typically polygonal, while DHov cells were more elongated and included small round cells; although both strains exhibited morphologic evidence of malignancy, no growth occurred upon injection into the anterior chamber of guinea pig eyes or the cheek pouch of cortisonized hamsters. Control inocula of HeLa cells also failed to grow. Again, the origin of these derived cells is not clear. Both cell types were susceptible to typical cytopathogenic effect of Types 1 to 7 adenoviruses, Types 1–3 polioviruses, Type B 3 Coxsackie virus, Types 7 and 8 ECHO viruses, herpes simplex virus, Greer strain "CA" virus, chimp "rhinitis" virus, and measles virus, and gave titers differing little from those obtained with HeLa cells. Propagation was demonstrated for representative viruses in this group. Specific destruction was not induced by influenza or mumps viruses, or by nasal secretions from subjects with common colds [Jordan (144)].

Many other cell strains of varying origin and character have been described [Hull (66); Bang & Gey (145); Graham & Siminovitch (146); Osgood & Brooke (147); Perry *et al.* (148); Bell & Johnson (149); Foley & Drolet (150); Haff & Swim (151)] but their potentialities for virus research have not been delineated by publication of complete spectra of viral susceptibility.

Biology, nutrition, and biochemistry of uninfected cells.—The intimate relationship between cell and infecting virus makes thorough knowledge of the biology of uninfected cells in culture essential to the research virologist. Requisite information includes normal cellular responses to nutritional and cultural conditions, so that (*a*) virus studies can be carried out with host cells of known physiological state, and (*b*) cellular responses to virus infection can be interpreted properly. Among many biological studies done with cell cultures, a few will be cited to illustrate quantitative techniques directly or indirectly useful in virus research. Studies by Earle *et al.* (152, 153) on influences on culture growth of inoculum size and speed of rotation of roller drums suggested that oxygenation was a limiting factor in stationary surface substrate cultures of strain L mouse cells. Kuchler & Merchant (154) investigated growth kinetics of strain L suspended-cell cultures, and showed that these mammalian cells were similar to bacteria in their growth behavior. Precise investigation of the genetics of viruses infecting mammalian cells was made possible by the cell plating technique devised by Puck, Marcus & Cieciura (79): various biological properties of HeLa cells were defined, including division time, influence of serum type on colonial morphology, plating efficiency and generation time, and average cell areas and volumes. Definition of survival curves for irradiated cells [Puck & Marcus (155)] should aid irradiation studies of viral genetics. Use of plating efficiency to expose nutritional mutants in parental cell populations [Puck &

Fisher (156)] has possible application to investigation of the influence of cellular enzyme systems on virus reproduction. Colonial growth of epithelial cells derived (62) from Chang's (132) strains of normal human conjunctiva, liver, kidney and appendix showed the general applicability of plating technique.

The state of continuously cultivated human or other cells with respect to malignancy interests virologists as well as cancer biologists. Although stable or clonal cell strains may be superior to mixed cell cultures from tissue for production of vaccine virus, their use has been avoided because of the remote hazard of cancer transmission to inoculated humans. A biological test of malignant state is desired because (*a*) cells of long cultivated strains show somewhat suggestive histological signs of malignancy; (*b*) appearance of histological evidence interpreted as indicative of malignancy during subcultivation of normal human cells has been reported [Leighton, Kline & Orr (157)]; and (*c*) *in vitro* conversion of normal to malignant cells by biological criteria has been described [Earle *et al.* (118); Gey (158); Goldblatt & Cameron (159)]. Sanford *et al.* (160) reported variation in malignant behavior even among clonally derived cells in culture. Attempts have been made to investigate malignancy of human cells of established strains by cytological and chromosomal analysis, and by inoculation into irradiated and cortisonized weanling rats and into human volunteers in late stages of malignant disease [Moore, Southam & Sternberg (161)]. As yet, neither biological nor biochemical [Leslie, Fulton & Sinclair (162)] tests can be considered satisfactory for diagnosis of malignancy of cultivated human cells.

Immunologic tests have not been employed for identification and differentiation of cultivated cells, but demonstrated cytotoxicity of antisera [Imagawa, Syverton & Bittner (163); Mountain (164); Miller & Hsu (165)] suggests feasibility of such use. Immunological application of cell cultures in virus research was suggested by work of Mabry *et al.* (166, 167): they observed an accelerated phagocytosis of virus-treated erythrocytes by cultivated macrophages which might have use as an erythrophagocytic test for diagnosis of certain virus diseases.

Discussion of nutrition will be limited to cells of known established strains since these seem the most likely candidates for use in investigation of virus biochemistry. Attempts to define nutritional requirements of cells in continuous culture have followed two courses; (*a*) empirical evolution of satisfactory growth medium, followed by stepwise elimination of unnecessary components, and (*b*) stepwise synthesis of growth medium by addition of defined components to a survival medium containing a minimal amount of dialyzed serum. Valuable contributions of Canadian workers [Morton, Morgan & Parker (168); Healy, Fisher & Parker (169); Morgan & Morton (170); Morton, Pasieka & Morgan (171)] have been acknowledged in reviewing use of synthetic mixtures for growth and maintenance of cells. Using quantitative techniques, Sanford *et al.* (21) showed that the growth-promoting power for strain L mouse cells of complex medium resided primarily in residue of horse serum and ultrafiltrate of embryo extract. Com-

pared with natural media, two unsupplemented defined solutions were found (172) to have little capacity to maintain cells. The amino acid component of mixture 199, however, when added to washed horse serum residue and embryo extract ultrafiltrate, fulfilled the minor growth-promoting function of horse serum ultrafiltrate (173). Later it was shown by Sanford *et al.* (174) with strain L cells that both globulins and albumins, when carefully separated from whole horse serum, could be substituted for horse serum residue to yield comparable rates of cellular multiplication. Kent & Gey (175) confirmed actual utilization of native proteins by cultivated cells: electrophoretic analyses demonstrated significant depletion of alpha and beta globulins in serum medium used for growth of two strains of malignant rat cells and the A.Fi. human fibrosarcoma strain over five and ten-day periods. Starting with an arbitrarily constituted defined medium supplemented with dialyzed horse serum which permitted multiplication of strain L cells, Eagle (176) defined 12 essential amino acids; on omission of any one, the cells were stated to undergo cytopathologic changes which were temporarily reversible. Concentrations of the essential L-amino acids defined as optimal were mostly less than those found in mouse plasma. All D-isomers were inactive but did not inhibit the active L-forms. The essential number included three not required for adult mouse growth. Procedures used for L mouse cells yielded similar results with HeLa human epithelial cells (177): optimal concentrations and essentiality of the same amino acids required for L cells were defined. The number included three more than was required for maintenance of nitrogen balance in man. Needed concentrations as evaluated are found in human serum. Seven known vitams proved essential for survival and growth of both strain L and HeLa cells in culture (178); the fact that cells in normal culture accumulated considerable reserves of vitamin components could account for failure to demonstrate more extensive needs. Some complexed vitamins (FMN, DPN, cocarboxylase) were as active, while others (FAD, TPN, coenzyme A, pyridoxal phosphate) were less nutritionally active than counterpart vitamins (179). In media lacking one of the 12 amino acids essential for strains L and HeLa, cells grew normally on addition of appropriate dipeptides (180). Definition of ion (28) and L-glutamine [Eagle *et al.* (181)] requirements produced media permitting continued growth of L and HeLa cells on supplementation with less than one per cent of serum. With two clonal strains of mouse liver parenchymal epithelial cells, Westfall *et al.* (182) also showed glutamine need, and reported an interesting difference in rate of glucose utilization and lactic acid production between the two cell lines. Of 19 cell strains tested [Eagle *et al.* (183)] varying widely in type of cell and species origin, 16 strains showed different but definite requirement for inositol, one (L 929 mouse fibroblast) was indifferent, and two (HeLa and J 111 human leukemia) were inconsistent in their need. Similar nutritional studies are being carried out with other cell strains; individual differences in cellular requirements are exemplified by the findings of McCoy *et al.* (184) with a stable strain of mouse carcinosarcoma, Walker-256. The Walker cell required the 12 amino acids and glutamine essential for

growth of L and HeLa cells, required asparagine in addition, and, unlike HeLa cells, it was stimulated to increased growth by nonessential glycine or serine.

A good beginning has been made on elucidation of the biochemistry of cultivated cells. Again, attention will be paid to well-known cell strains, for reasons of economy rather than intent to deprecate considerable information gained from study of primary cultures. Siegel & Cailleau (185) found respiration of strain L cells increasingly inhibited by increasing concentrations of iodoacetate and sodium azide, and first stimulated, then suppressed, by increasing 2,4-dinitrophenol. With strain L cells, Pace & Phillips (186) demonstrated growth inhibition by 10^{-4} *M* sodium cyanide, but some stimulation of growth occurred at a low cyanide level; about 15 per cent of respiration persisted despite a cyanide concentration slightly stronger than necessary to stop growth. Further studies on metabolism by Phillips & Feldhaus (187) were comparable to those on cyanide sensitivity in showing that L cells metabolize like most normal tissue. Unlike the metabolic properties described by Burk for malignant cells (188), L cells exhibited moderate respiration, a respiratory quotient of unity, low anaerobic and aerobic glycolysis, and great stimulation of respiration by *p*-phenylenediamine. Although it has been commonly conceived that animal cell constituents exist in dynamic equilibrium by continual regeneration, Siminovitch & Graham (189) found conservation of both ribonucleic acid phosphorus and deoxyribonucleic acid phosphorus in L cells growing logarithmically in suspension cultures. Biochemical analysis by Westfall *et al.* (190) of serum-egg-ultrafiltrate medium used for suspension cultures of HeLa cells suggested that new cells had been synthesized more from serum protein than from free amino acids. Phillips & McCarthy (191) reported that HeLa cells grown in human serum exhibited greater respiration and glycolysis than cells propagated in horse serum medium. Stock cultures were carried in 10 per cent human or horse serum diluted in salt solution containing 0.025 gm. per cent each of yeast and lactalbumin hydrolysate. Respiration was low, aerobic glycolysis moderate, anaerobic glycolysis, low, and *p*-phenylenediamine stimulation of respiration high; there was no Pasteur effect. Sonically disintegrated cell-free extracts of HeLa cells grown in Eagle's medium supplemented with serum protein were reported by Barban & Schulze (192) to contain all the enzymes of the Krebs' citric acid cycle. Presence of soluble alpha-ketoglutarate oxidase, L-malic dehydrogenase, and "L-malic enzyme" was also demonstrated. Leslie, Fulton & Sinclair (193) studied the biochemistry of HeLa cells cultivated in medium 858 containing 20 per cent human serum and 5 per cent chick embryo extract: measurements of glucose consumption, lactic acid and total keto acid production, and cellular DNA phosphorus, indicated a disproportionate relation between cell number and glucose consumption. Cellular numbers were, however, linearly related to the amount of glucose consumed but not accounted for as lactic and keto acid. These same authors observed that HeLa cells maintained in medium 858 with 20 per cent human serum on exposure to insulin responded with increased synthetic activity and glucose

utilization. Apparently, this response represented accelerated but not altered metabolism, in contrast to the response of human skin fibroblasts which oxidized a greater proportion of consumed glucose under the influence of insulin. Two reports exemplify procedural aids developed for biochemical investigation with cell cultures. Oyama & Eagle (48) described use of the Folin-Ciocaltau reagent for analysis of protein as a measure of celluar growth, and conversion factors for seven human and one mouse cell strains. Harris (194) reported an improved microscope chamber incorporating an oxygen electrode for simultaneous observation of cellular multiplication and respiration. The apparatus appears potentially valuable for physiological study of virus infection.

Applications to Virus Research

Isolation, propagation, and titration of viruses.—Research sponsored by The National Foundation for Infantile Paralysis, Inc., has, in the past six years, accelerated development of cell culture systems for detection and growth of animal viruses. The *in vitro* system, with advantages of economy, simplicity, and amenability to control over the embryonated egg or intract animal as an experimental host of viral infection, facilitates virus detection and titration. The continuing development of *in vitro* systems has made less evident contributions to medical virology in evolving simpler and better defined concepts of experimental virus infection. The concepts are simpler, not because complexity has been eliminated, but because component factors are inherently susceptible to control and rigorous definition as they are not with the intact animal system.

Viruses now are commonly isolated from natural sources by use of monolayer cell cultures on glass substrate in tubes or bottles. Accepted criteria of virus infection and propagation include: (*a*) distinctive cytopathologic effect neutralizable by specific antiviral serum; (*b*) maintenance of infectivity despite serial cell culture passage to achieve a cumulative dilution significantly in excess of the titer of original virus; and (*c*) significant increase in virus content during a single cell culture passage. Use of cell cultures for isolation and titration of a particular virus requires (*a*) quantity production of cellular populations uniformly responsive to virus infection, and (*b*) methods for preparation of viral inocula from source material so as to render them free of microbial contamination or toxic effect or both. Appreciation of the extraordinary usefulness of antibiotics for control of microbial contamination should be tempered with realization that cell cultures can carry some bacterial infections unnoticed unless adequate sterility tests are done. Although, in a few special cases, completely defined media have not been developed for indefinite maintenance of cells in culture, the large reserves of essential nutrients accumulated by animal cells in culture permit maintenance of established cells in relatively simple defined solutions (31). Some aspects of the question of suitability of mixed cellular populations from tissues versus more homogeneous populations of established cell strains for study of virus have already been reviewed. In addition, it should be noted

that relatively homogeneous cellular populations and rigidly standardized techniques for cultivation and maintenance are necessary for selection of a cell system of maximum sensitivity and usefulness for study of a particular virus, if the complicating factors apparent in earlier work (195) are to be avoided. Comparison of the efficiency of cell systems or culture techniques for isolation of virus from natural sources can be made on the basis of percentage recovery of virus in parallel tests on the same specimens [Kibrick, Enders & Robbins (196)]. While comparisons based on arbitrary procedures may be useful during early development of cell cultural methods, precise evaluation of relative cellular sensitivity should be based on prior definition of optimum conditions for each cell type including form of culture, nutrient medium, cellular physiological state, virus inoculum, number and condition of viable cells exposed to virus, and temperature of incubation. Careful study may provide alternate cell systems of equal sensitivity and convenience. The value of cell culture methods for medical diagnosis as well as epidemiological virology (18, 123), is exemplified by a representative study of hospital patients made by Godenne & Riordan (197); combined results of complement fixation and cell culture neutralization tests were diagnostic in a majority of poliomyelitis infections proved by virus iolation. The problems attending differential diagnosis of poliomyelitis have been indicated by Rhodes, Wood & Duncan (198) in a comprehensive discussion of laboratory methods with emphasis on tissue culture protocols.

The value of cell cultures in supplementing or replacing less expensive animals than monkeys for epidemiological research is shown, for example, by studies of Beale *et al.* (199) on the role of Coxsackie virus in aseptic meningitis. Successive developments with Coxsackie viruses that promise understanding of the role as cytopathogenic agents also are indicative of the continuing technical improvement of cell culture methodology. Initial cultivation *in vitro* of Coxsackie virus in Maitland-type culture [Slater & Syverton (200); Weller *et al.* (201)] was supplanted by use of primary explant cultures of such tissues as mouse interscapular fat [Stulberg *et al.* (202)]; monkey testicle [Riordan *et al.* (203)]; and skeletal muscle [Stulberg *et al.* (204)]. Differences in cellular susceptibility aiding differentiation of virus type was observed. Later, diagnostic methods were developed for isolation of cytopathogenic group B Types 1 to 4 Coxsackie viruses from fecal specimens, and for typing by use of trypsinized monkey kidney cell cultures for neutralization tests [Sickles *et al.* (205)]. HeLa cells were found sensitive to cytopathogenic propagation of several Coxsackie strains, including a B 1, six B 3, a B 4, and a "Texas-14" virus [Crowell & Syverton (128)], later established as a mixture of A 14 and B 3 strains.

Cell culture methods have been instrumental in lengthening of the list of known animal viruses, as is illustrated by the history of adenovirus recognition. Using HeLa cells, Hilleman & Werner (206) isolated a new virus not infectious for common laboratory animals or embryonated eggs, which apparently was responsible for certain cases of acute pneumonic or respiratory disease occurring in an influenza epidemic among service personnel.

Other similar but immunologically separable viruses recovered from spontaneously degenerating human tissue cultures, body secretions and excretions, or specimens from acute respiratory disease [Rowe *et al.* (207, 208)], made up a new collection of viruses known as the APC (adenoidal-pharyngeal-conjunctival) or adenovirus group (209). These viruses are characterized by lack of pathogenicity for laboratory animals, unique cytopathogenic effect in certain human and animal cell cultures, and production of a common complement-fixing antigen. Adaptation of adenoviruses to passage in monkey kidney cultures [Hartley *et al.* (210)] has provided a basis for production of vaccine for human use [Bell *et al.* (211)].

Cell culture methods were wholly responsible for recognition of the ECHO (enteric cytopathogenic human orphan) viruses (131). These new agents were first discovered by cytopathogenic effect on human and monkey cell cultures not neutralizable by known antiviral sera, and subsequently are being assoicated with human disease [Karzon *et al.* (212)]. Other orphan viruses have been isolated as a consequence of inapparent infection of animal donors for cell cultures [Enders & Peebles (213); Rustigian *et al.* (214)], or of contamination of clinical specimens or natural cell culture media [Hull *et al.* (215)]. The action of these agents is annoying in that they can render cultures unusable for other purposes, confuse the results of virus isolation and neutralization tests and be inadvertently incorporated in human vaccines; contrariwise, their discovery is evidence that tissue culture of biopsies from patients with etiologically uncharacterized disease may be useful for recovery of agents not found in body secreta or excreta.

In vitro methods for epidemiological and diagnostic studies of known viruses continue to be expanded. Examples are seen in cultivation of dengue virus in rhesus monkey testicular and kidney cell cultures [Hotta & Evans (216)]; Sindbis virus in chicken embryonic, human adult uterine, or monkey testicular tissue cultures [Frothingham (217)]; and influenza, herpes, Newcastle disease, and vaccinia viruses in one or another culture of HeLa, chick embryo, human embryonic lung or kidney, or monkey kidney cells [Stulberg & Schapira (218); Tyrrell (219); Mogabgab *et al.* (220, 221)]. Herpes viruses have been studied by use of cells from rabbit peritoneal exudate [Barski *et al.* (222)], trypsinized rabbit kidney [Barski *et al.* (223); Sosa-Martinez *et al.* (224)] or explanted rabbit corneal and human kidney epithelium [Doane *et al.* (225); Beale & Hair (226)]. Roller tube cultures of human embryonic skin, muscle and foreskin, as used by Weller & Stoddard (227) and Weller (228), permitted combined use of cell culture and fluorescent antibody techniques for definitive study of varicella infection [Cheatham *et al.* (229)]. Peculiar lesions neutralizable with convalescent serum appeared in cultures of adult monkey and human embryonic lung or other tissue inoculated with throat washings from disease diagnosed as rubella, suggesting cultivation *in vitro* of another virus otherwise difficult to study because of animal refractoriness [Anderson (230)]. Enders & Peebles (213) reported isolation in cultures of various human tissue and monkey kidney of several viruses from blood and throat washings of measles patients, producing cytopathogenic

effects neutralizable by convalescent serum; convalescent measles serum also reacted specifically with antigenic material produced in cultures of these viruses. The Edmonston strain of measles virus so isolated and carried in human kidney cell cultures (213) was passed to Eagle's strain KB human epidermoid carcinoma cells, with similar cytopathogenic effect and production of specific complement-fixing antigen [Dekking & McCarthy (231)]. Black *et al.* (139) found that this strain of measles virus could also be adapted to propagation in Hep-2 human carcinoma cells. Mumps virus passed a few times in chick embryos or obtained directly from acute parotitis was readily propagated in monkey kidney epithelial or HeLa cell cultures [Henle & Deinhardt (232)]; diagnostic evidence was obtained earlier by neutralization with convalescent serum than by hemagglutination inhibition with chick embryo virus. Cytopathogenic effect of indigenous simian virus, resembling that of mumps virus, reduced usefulness of monkey kidney cultures compared to HeLa cultures.

Various poorly characterized or merely suspected viral agents of human disease are now being studied with the aid of cell cultures. Propagable viruses cytopathogenic for Detroit-6 cells are claimed to have been recovered from sera or stools of patients apparently suffering from infectious hepatitis [Rightsel *et al.* (233)]. Species-specific salivary gland viruses are harbored inapparently by man, monkey, and rodents to provoke, exceptionally, a fatal generalized infection and production of giant cells with large intranuclear inclusions. The generalized infection in infants, known as cytomegalic inclusion disease, is contracted *in utero*. Smith (234) made this virus available for study *in vitro* by propagating it in human uterine fibroblast cultures, with production of cytologic changes indistinguishable from those produced in mouse tissue by mouse salivary gland virus (235). Similar indigenous viruses were recovered by Rowe *et al.* (236) from spontaneously degenerating human adenoid fibroblast cultures, a complement-fixation test was developed with culture material, the incidence of complement-fixing and neutralizing antibody in infant and adult sera studied, and serological relationship established between one strain and viruses recovered by other workers. Chang's human liver epithelial cell strain was employed for isolation of a cytopathogenic virus etiologically involved in a coryza epizootic among chimpanzees [Morris *et al.* (237)] but nonpathogenic for the usual laboratory animal hosts. The agent was retransmissible to chimpanzees with production of clinical symptoms and specific antibody. The virus was serologically implicated in a case of human laboratory infection. General human experience was indicated by the presence in human sera of complement-fixing antibodies to the new virus, and failure to neutralize its cytopathogenic effect with antisera to several new or recently propagated human and simian viruses. Viruses distinctively cytopathogenic for cynomolgus monkey kidney epithelial cultures were isolated by Chanock (238) with results suggesting etiologic relationship to the syndrome called "viral croup." Properties of these viruses were such as to associate them with the myxovirus group (239);

distinction from other group members was indicated among other ways by production of an unusual hemagglutinin.

Veterinary medicine has benefited equally from the advent of cell culture methods for the study of old and new viruses. Chanock (240) indicated suitability of monkey kidney cultures for isolation and propagation of Newcastle disease virus, which was recovered from degenerating explant cultures of chick embryo fibroblasts unknowingly embedded in contaminated chicken plasma. With monkey kidney cells, infectious titers were moderately high and hemagglutinin titers low compared to those of infected allantoic fluid. Cytopathogenic effect of Newcastle Disease Virus (NDV) for other cell types was reported by Tyrrell (219), Pereira & Gompels (241), Fastier (242), and Mason & Kaufman (243). NDV has been adapted to propagation in Ehrlich ascites tumor cells maintained *in vitro* (these cells apparently did not form true cultures), with parallel increase in propagability and oncolytic effect on similar cells *in vivo* [Flanagan *et al.* (244)]. Nearly equivalent yields of vesicular stomatitis virus have been obtained by propagation in surviving suspensions of trypsin-dispersed guinea pig and bovine kidney cells, or in grown cultures of guinea pig epithelial and fibrocytic cells [Bachrach *et al.* (245)]. The same authors were able to use mixed epithelial and fibroblastic outgrowth of trypsinized porcine or bovine kidney for propagation, titration, neutralization, and large-scale production of Type A foot-and-mouth virus (246). Attempts to isolate in laboratory animals an etiological agent responsible for outbreaks of bovine rhinotracheitis occurring in California and Colorado since 1951 were unsuccessful until cell culture procedures were used; isolation, assay, and neutralization of a virus cytopathogenic for beef embryo kidney, testicle, and lung cultures but not for human or chick cells were accomplished, and characteristic symptoms reproduced in calves with tissue culture material [Madin *et al.* (247)]. Previous work on tissue culture propagation of infectious canine hepatitis virus (248, 249) has been continued by Fieldsteel (250). He has used canine renal epithelial monolayer or suspended cell cultures for isolation, propagation, and assay of virus, serologic diagnosis by neutralization or complement fixation, definition of cellular range *in vitro*, and reversible modification in cell culture of virus virulence.

Finally, a use of cell culture minor in frequency but major in value is developing: study of tumorigenic viruses. Pikovski (251) reported apparent propagation of RIII mouse mammary tumor virus in plasma clot cultures of chicken fibroblasts. Rubin (252) showed that Rous sarcoma virus could be harvested from supernatant fluid of sarcoma cell cultures established in plasma or on glass. Evidence for the propagation of fully infectious cottontail rabbit fibroma virus in mixed epithelial and fibroblastic cultures of immature cottontail testis, with production of acidophilic intracytoplasmic cellular inclusions, was presented by Kilham (253). Continued replication of fibroma virus in domestic rabbit testicular cultures was not obtained, and only limited survival occurred with cultures of monkey kidney and human foreskin.

This abbreviated selection of references illustrates the power of cell culture in revealing viral trophisms, in providing sensitive methods for isolation of new viruses and their propagation in homologous tissue without need for and less chance of adaptive change, and in permitting use of convenient "hosts" for titration or production of virus in volume.

Visualization of effects of virus infection at the cellular level.—Studies of cytopathology of virus infection *in vitro* up to 1954 have been reviewed by Enders (254); some aspects have also been discussed by Lynn & Morgan (255), Pollard *et al.* (256), Moore (257), Dulbecco (258), and Syverton (259). An aspect of virus-induced cytopathology of paramount investigative importance is that of plaque formation, since this makes quantitative virology possible. The variety of vessels, viruses, and cell types with which viral plaques have been demonstrated [Hsiung & Melnick (56); Takemori *et al.* (260); Ledinko (261); Granoff (262); Fogh & Lund (263)] suggests that this quantitative assay method can be applied with any virus-cell system capable of exhibiting visual cytopathogenic effect. The procedure even appears applicable when discernible cellular abnormalities appear without plaques of cellular destruction, according to the observations of Manaker & Groupé (264), relating dilution of Rous sarcoma virus to counts of foci of altered morphology in chick embryo cell monolayers. The value of plaque count technique with animal cell-virus systems for investigation of the biology of virus infection at the cellular level is demonstrated by such studies as those of Dulbecco & Vogt (265) and Lwoff *et al.* (266). Plaque analysis permits (*a*) estimation of the minimal number of independent units capable of infecting a cell; (*b*) determination of relation between such units and the physical particles visualized by electron microscopy; (*c*) derivation of clonal lines of parent or mutant viruses; (*d*) delineation of extra- and intracellular virus growth curves; and (*e*) observation of the effect of physical and chemical agents on viruses and other features of virus biology, with a precision not otherwise obtainable. As previously described [Syverton (259)], cytopathic cellular alterations include at least five categories: (*a*) final total destruction, typified by the cytopathogenic effect on both epithelial and fibroblastic cells of polioviruses, and some Coxsackie and ECHO viruses; (*b*) incomplete degenerate changes revealed by a mixture of necrotic cells and shrunken cells of altered staining characteristics including nuclear pyknosis, typified by effects of Newcastle disease virus and arthropod-transmitted encephalitogenic viruses; (*c*) effects similar to those produced by viruses of the vaccinia-herpes-B-pseudorabies group, seen as an outward spreading development of typical cellular inclusion bodies surrounding enlarging foci of cellular degeneration; (*d*) clumping and detaching of cells from glass without cessation of metabolism, characteristic of adenovirus infection of both epithelial and fibroblastic cells; and (*e*) focal degeneration of cells evidenced by the appearance of multi-vacuolated cytoplasm and formation of giant cells as induced by measles and simian viruses. These morphologic changes which with experience are recognizable as distinctive for particular viruses, are the basis of

dilution titrations *in vitro* and tentative identification of viruses. It must be emphasized, however, that use of cell cultures for identification and assay of viruses depends on careful use of adequate cell controls and neutralization of cytopathogenic effects with appropriate antiserum. For other than these practical uses, cell culture has value in facilitating examination of the cytopathologic effect of virus on particular cells [Hogue *et al.* (267)]; study of virus toxins [Wagner (268)]; definition of the range of animal host cells susceptible to a virus [Mason & Kauffman (243)]; elucidation of the pathologic relation between replicated virus and host cell [Enders (269)]; provision of additional characters for differentiation of related viruses [Ledinko, Riordan & Melnick (270)]; and suggestion of mechanisms by which virus may disseminate through tissue from cell to cell [Black & Melnick (271)].

Some aspects of poliomyelitis research illustrate current emphasis on cellular aspects of viral cytopathology. By employing metabolism-blocking agents, Ackermann *et al.* (272) were able to examine the time relation between virus synthesis and development of cellular injury, concluding that the latter was at least semi autonomous in that it influenced viral yield rather than the reverse, and that morphologic alterations might reflect metabolic damage occurring as a side effect of the infectious process. In continuation of previous studies of cytopathic alteration of an adult human fibroblast infected with Types 1 or 2 poliomyelitis virus, Barski *et al.* (273), by means of continuous phase contrast cinemicrography, noted progressive formation of a dense motionless mass in paranuclear cytoplasm; as cytoplasm was consumed to form the dense body signs of nuclear damage appeared, the cell exhibited violent "bubbling," the remaining marginal cytoplasm became vacuolated, and the infected cell gradually disintegrated. Fogh (274) found that monkey kidney cells absorbed a constant fraction of poliovirus over a broad range of exposure multiplicity. He observed a linear relation between the log of exposure multiplicity and the time required for development of defined stages of cellular damage; this relation could be used for titration purposes. By arbitrarily describing four stages of cytopathology and counting numbers of typically altered cells, Dunnebacke (275) for poliovirus Types 1–3 infections related virus release to similar progressive damage to monkey kidney, HeLa, and a variety of human fetal cells; she suggested that nuclear injury precedes the appearance of fluid-phase virus and that virus release occurs in the interval between formation of the paranuclear cytoplasmic body and cellular disintegration. About 95 per cent of finally released virus appeared in the medium after 50 per cent of the cells had passed through three of the four stages of damage. In contrast to these findings, similar studies with poliovirus-infected human amnion cells (276) showed a different sequence of morphological alteration and virus release. This and like research (277 to 280), while involving the usual difficulties attending the relation of visible effects to physical events, is representative of continuing efforts by many workers to elucidate physical details of the cell-virus relation. It is not apparent whether morphologic damage is a specific consequence of virus

synthesis or is indirectly mediated, say, by diversion of energy needed for cellular maintenance. As shown by Henle *et al.* (281), production of complete infectious virus is not necessarily prerequisite for cytopathogenicity. HeLa cells damaged after exposure to large doses of influenza virus synthesized new hemagglutinins and both particulate and soluble complement-fixing antigens, but did not produce infectious virus. These results, as well as casting light on phases of virus reproduction, emphasize that cytopathic change of the cell is insufficient evidence of the capacity of a particular cell type to support multiplication of infectious virus.

Cell culture systems allow combined application of various visualization techniques for study of the mechanics of viral reproduction and effect on host cells. As Reissig & Melnick (282) have pointed out, and as the continuing studies of Gey & Bang and their associates (145, 283, 284, 285) have so effectively demonstrated, monolayer cell cultures offer a system which permits correlation of changes in infected cells seen by light or phase microscopy with (*a*) development of physical virus particles observed by electron microscopy, and (*b*) with replication of infectious virus. Particularly with clonal lines, cultivated cell populations are relatively uniformly susceptible to virus, are monodispersible for relative synchrony of infection, and can be used for enumeration of infective virus particles. With such a system ,the necessarily small samples subjected to visual examination can be made properly representative of succeeding stages of virus infection. Monolayer cultures can be established in vessels or chambers with walls of optical quality suitable for continuous examination by phase microscopy, on glass coverslips for cytological examination of stained specimens, or on coverslips coated with resin film which can be detached together with the adhering cellular monolayer for ultrathin sectioning or direct insertion in the electron microscope. Details so revealed of the structure of cells and of developmental virus forms are seen in pictures such as those of Reissig & Melnick (282) showing sequence of morphologic alterations and formation of particles accompanying herpes B virus infection of monkey kidney cells. Formation of intranuclear inclusions as seen by ordinary microscopy was correlated with the appearance of particulate components seen by electron microscopy, and with increase in content of infectious virus in the culture fluid. Points of procedure for preparation of cell culture specimens for electron microscopy apparently can be varied. For their studies of HeLa cells infected with adenoviruses, Harford *et al.* (286) at first detached HeLa cells from the glass of growth tubes by incubation with citrate solution, but finding that cells so removed and embedded for sectioning with glass knives contained dulling material they subsequently fixed and further treated cells while still attached to glass. For embedding, a small spatula was employed to remove treated cells from the glass substrate. Electron microscopic examination was combined with an approach to cytochemical inspection by use of companion infected cultures stained for DNA, lipide, and mitochondria. Intranuclear particles observed in infected cells were tentatively identified as virus by reason of regular structure and arrangement. Since most cells exposed to

virus exhibited cytopathic alterations while only a small number contained the virus-like particles, it was suggested that the cytopathic effect of adenoviruses on HeLa cells is related to toxicity rather than virus reproduction. Apparent confinement of these virus particles to nuclei of affected cells indicated a possible reason for the usually low yield of virus *in vitro*. A third technique, the immunocytological, can be brought to bear on the problem of morphological evolution of virus infection. Use of fluorescein-tagged antibody provides the necessary biological link between stages of particle formation seen by electron microscopy and development of morphologic alteration delineated by cytochemical or cytological examination of stained cultures. Lacking an experimental host to establish identity of agents obtained from varicella or herpes zoster and carried serially in culture as a propagable focal cytopathogenic effect, Weller & Coons (287) generalized Watson's (288) use of fluorescein-conjugated antiviral serum by employing dye-tagged rabbit antihuman gamma globulin serum to detect specific combination of intracellular virus with homologous human untagged antibody. Evidence supporting propagation *in vitro* of varicella and herpes zoster viruses was supplied by specific reactions of convalescent serum specimens with infected human cell cultures. As observed by Noyes (289) with a strain of human epidermoid laryngeal carcinoma cells infected with the neurotropic Egypt 101 virus, fluorescent antibody can be used to make microscopic plaques of virus-induced degeneration visible as a measure of virus concentration in inoculated material. A variety of similar studies (290 to 293) confirm the value of the fluorescent antibody technique in conjunction with cell cultures as a means (*a*) for identification of a human disease agent not detected by propagation *in vitro* or through cytopathogenicity; (*b*) for correlation of appearance of intracellular viral products with material entering culture fluid and detectable by complement fixation or neutralization test; (*c*) for demonstration of association between virus material and intracellular inclusions as seen by ordinary microscopy; (*d*) for study of the influence on cellular mitosis of viral infections; (*e*) for analysis of the cell-to-cell mode of virus spread or mechanism of virus release or both; and (*f*) for clarification of such intracellular events as the apparent passage of virus from nucleus to cytoplasm.

Qualitative and quantitative virus neutralization and typing, and epidemiological application.—In recent years, cell cultures have almost wholly replaced explant cultures, animals, and chick embryos for neutralizing antibody assays. The most significant development, however, has been the establishment of adequate theoretical bases of the neutralization test *in vitro*. While qualitative assay of serum neutralizing antibody can be performed *in vitro* either as a protection test (by addition of serum directly to cultures prior to challenge with the test virus) or as a neutralization test (by inoculation of cultures with test virus already incubated with serum antibody), the latter form most commonly is employed. Likewise, it is now generally agreed that the serum-dilution test giving a PD_{50} (50 per cent protective dose) titer as a measure of serum antibody has greater practical value than the virus-

dilution test resulting in a neutralization index as a measure of the antibody. Development of a neutralization assay for antibody to a particular virus is an immunological rather than a cell culture problem, since cultures are assumed to function only as hosts for assay of residual virus. As with animal hosts, diagnostic use of cell culture neutralization properly requires testing of dual specimens of a patient's serum taken during the acute and convalescent phases of disease. Pertinent to the problem is recognition that test virus preparations obtained from cell cultures and used as antigens for hyperimmune typing serum are also suspensions of cellular material which may induce formation of cytotoxic as well as antiviral antibody; the neutralization test always should include undiluted serum controls as well as uninoculated cell controls. The choice between virus-dilution and serum-dilution procedures for assay of neutralizing antibody should be made on the basis of box titrations of test virus and representative test sera. The virus-dilution procedure, while usually requiring a greater number of indicator tube cultures, has the advantages (*a*) that the test dilutions for many viruses can be prepared and stored frozen for subsequent incubation with single amounts of test and normal sera, and (*b*) that the test dose of virus is not a factor in the determination of serum titer as it is with the serum dilution procedure. For a useful application of the latter procedure, regression of neutralized virus on serum protective titer should be such that a five fold variation in the amount of virus added to serum dilutions is accompanied by a twofold or less alteration in serum neutralization titer, when tenfold virus dilutions are tested against twofold serum dilutions. Since the virus-antibody combination can be reversible, time and temperature of virus-antibody incubation, time required for maximum adsorption of unneutralized virus by indicator cell cultures, and the extent of dilution dissociation of virus-antibody complexes are factors which must be considered in the development of a neutralization assay. Antigen-antibody dissociation may account for earlier reports of virus "breakthrough" in some neutralization tests. Statistical advice may be helpful in deciding the relative merits of 50 versus 100 per cent protection endpoints, selection of efficient serum-dilution intervals, and allocation of tubes per dilution for a limited number of available tube cultures. When large-scale antibody assays are contemplated, adequate experimental and statistical design is essential to the economical employment of cell cultures. When 50 per cent protection endpoints are used and statistical advice fails to provide a satisfactory answer to the problem of dilution choice and culture allocation, common sense indicates that use of a smaller number of tubes per dilution and dilutions spaced sufficiently close that at least one serum dilution must result in less than 100 and greater than 0 per cent protection, is superior to the use of a greater number of tubes per dilution and dilution intervals so broad as to miss the area of partial protection.

When cell cultures are substituted for animals in virus-neutralization tests, it may be desirable to compare results. Li & Schaeffer (294), for example, described a relatively simple cell culture procedure with results in agreement with mouse intraspinal inoculation for 90 per cent of human sera

tested against Types 1–3 polioviruses. Melnick (295) reported that cell culture neutralization could be employed profitably for comparison with complement-fixation tests to provide a possible explanation of human heterotypic antibody response, and epidemiological correlation between population incidence of Type 2 antibody and immunity to Type 1 or 3 poliovirus. Monkey kidney cell cultures were used for the neutralization tests and to provide complement-fixing viral antigens. Of 70 strains of poliovirus newly isolated, about half, upon inactivation by heat, were found ditypic or tritypic, with the major type by complement-fixation test in agreement with the type assigned by neutralization tests. Importance of the test virus dose to diagnostic use of the serum-dilution neutralization test has been emphasized by Ward *et al.* (296). By use of 1000 doses of test virus, on the average, to assay the antibody content of acute and convalescent-phase sera from polio patients, they demonstrated an incidence of diagnostic antibody increases in the serum of polio-infected patients not seen by other investigators using 30 to 100 doses of test virus. The work of Bartell & Klein (297) on the content of poliovirus-neutralizing antibody in domestic animal sera, indicates the importance of the type of serum added to media to maintain cell cultures for virus or antibody assays. The definitive experiments of Dulbecco, Vogt & Strickland (298), with Western equine encephalomyelitis and poliomyelitis viruses, demonstrate how quantitative cell culture techniques may be applied to the investigation of the kinetics and mechanics of neutralization. This work shows how titers determined by endpoint neutralization tests with cell cultures could be affected by the persistence of unneutralized virus and by carryover of antibody from the reaction mixture to the cellular indicator system. The sort of prior investigation needed to develop a neutralization test is illustrated by Ginsberg's (299) studies on the use of HeLa cells for identification of adenoviruses and measurement of type-specific antibody. Special problems may be encountered with neutralization tests for particular viruses. For instance, if the test sera should contain antibodies to cytotoxic activity of virus distinct from virus-neutralizing antibodies, pseudoneutralization could result from the effect of "antitoxic" antibody on infectious virus replicated. In their studies of mumps virus neutralization with HeLa cell cultures, however, Deinhardt & Henle (300) found (by washing indicator cultures treated with virus-serum mixtures) that apparently true neutralizing antibody was being measured by anticytolytic effect.

Viral epidemiology is concerned with the characterization of viral infection on a population rather than on an individual basis. Many principles pertinent to the application of cell culture to this branch of investigation were recognized in early studies with culture systems since superceded. Laboratory requirements for epidemiological studies of herd response to infection include methods for (*a*) isolation of virus from human or animal tissues or fluids; (*b*) identification of virus and allocation by immunotype; and (*c*) detection and assay of antibody present in population members. Essential for comprehensive epidemiologic studies is a cell system which is

senstive to infection by viruses in question, capable of economic mass cultivation in relatively simple medium, and capable of maintenance in good morphologic and metabolic condition for a period sufficiently long to ensure response to low concentrations of virus encountered in clinical specimens. Epidemiological research should be based on sufficient prior investigation to establish and to characterize biologically the cell culture tool. While qualitative tests with clinical specimens make known the comparative usefulness of cell culture systems [Wenner & Miller (301)] for the isolation of virus, adequate evaluation requires quantitation of each determining factor. As yet, the influence even of the number of exposed cells on relative viral susceptibility of cell types has not been adequately investigated. Epidemiologic value of cell cultures as an alternative to animal hosts was soon established in representative poliovirus studies [Robbins *et al.* (302)] and is now commonly accepted. The neutralization test essentially is the principal weapon of epidemiological cell culture, since procedures usually are aimed at direct isolation and concomitant typing of virus from specimens. A weakly cytopathogenic virus may be identified indirectly by the immunization of animals, followed by typing of antisera so obtained with the reference prototypical strongly cytopathogenic virus (203), and ancillary use of the neutralization test for assay of serum antibody. Requirements for an adequate neutralization test as stated by Ledinko *et al.* (107) are still applicable: (*a*) the cell culture system must permit quantitative reproducible titrations of virus and antibody; (*b*) the cytopathic effect of the virus in cell culture must be distinctive, and its specific inhibition by homotypic antiserum likewise must be clearly visible; (*c*) uninfected control cultures must not succumb to nonspecific degeneration; and (*d*) it must be possible to obtain unequivocal test readings before occurrence of virus "breakthrough" in the presence of antibody, if any occurs. Preparation of nontoxic virus specimens is particularly important in the epidemiological application of cell culture. Fecal specimens may prove troublesome in this respect; preparatory procedures usually include homogenization in water or salt solution containing antibiotics, followed by successive low- and high-speed centrifugation to rid specimens of extraneous material and microbial contaminants, and possibly to effect virus concentration. Ether extraction [Riordan *et al.* (203)] was employed formerly; pH indicator added to fecal homogenate has been found to reveal toxicity of microbial origin [Syverton *et al.* (35)] and injudicious usage of strong antiseptics for disinfection of receptacles. Other methods for circumvention of fecal toxicity have involved testing of increasing dilutions of material, increasing the concentration of tissue in exposed culture, or both [Youngner *et al.* (303); Lahelle (304)]. Other details of epidemiological application of cell culture systems in current use have been reviewed (18, 203, 305).

Cell or tissue culture has been widely used to speed epidemiological characterization of virus infection by rapid immunotype identification of multiple virus strains from a single epidemic period or season [Duncan

et al. (306); Hilleman *et al.* (307); Hilleman *et al.* (308); Rowe *et al.* (309)]. Frequently such distinction has been sufficient to provide highly suggestive evidence of the character and mode of spread of an outbreak of disease [Eklund & Larson (310); Miller & Kamitsuka (311)]. Ability to test for differences within virus types, however, would be helpful in definitely tracing rather than suggesting the origin and dissemination of infection [Beale *et al.* (312)], or in explaining puzzling variation in epidemiological behavior of a single virus type [McCarroll *et al.* (313)]. Resolution of such differences may be an epidemiologically useful consequence of cell cultural study of viral genetics. Although, as with poliomyelitis, economy from use of the cell culture technique has resulted in various epidemiological studies otherwise less feasible (314, 315), *in vitro* procedures have permitted even more useful concomitant and detailed study of the epidemiology of viruses unassociated with known infection [Honig *et al.* (316)]. Thus, it becomes possible to associate distribution of identified viruses with disease as well as the reverse, and this approach, in turn, can provide unsought information. For instance, Jordan *et al.* (317), in the course of sequential cell culture studies of respiratory virus infections in family groups, uncovered family infections with polio and Coxsackie viruses not recognized clinically. Simplicity of etiological and epidemiological researches afforded by refined cell culture technique may (*a*) facilitate association of a specific virus with infectious disease of undetermined etiology [Jawetz *et al.* (318)]; (*b*) aid recognition of a clinical syndrome as a specific infection [Morgan *et al.* (319)]; or (*c*) help the characterization of a particular viral infection as a definite disease entity [Bell *et al.* (320)]. The increasing power of laboratory techique is reversing the conventional approach to epidemiology as a study after the fact of epidemic disease: the capacity to discover viruses before as well as after the occurrence of specific disease has uncovered opportunist pathogens acquired during childhood [Ramos-Alvarez & Sabin (321, 322); Sabin (323)].

Quantity production of virus for physico-chemical analysis, serological assay, or vaccine manufacture.—Cell cultures, particularly of clonally derived strains should be excellent sources of virus material for purification by such methods as those recently employed successfully with polio and Coxsackie viruses [Schwerdt & Schaffer (324, 325); Hampton *et al.* (326); Mattern & DuBuy (327)]. Fluid from infected cell cultures in comparison with tissue-fragments suspensions (325) should provide source virus representing a greater proportion of medium protein since, with cells in monolayer or suspension cultures, the total potential capacity for virus production can be more nearly realized. As more completely defined media for growth and maintenance of cells are developed, it should be possible to reduce still further the nonviral protein content of culture fluid.

A somewhat different problem is encountered in use of cell cultures for preparation of complement-fixing viral antigens. For routine diagnostic use of the complement-fixation test, a standard tube procedure is preferable: complement fixation of this order usually calls for viral suspensions with

titers of 10^8 50 per cent infectious doses per ml. or greater. The obstacle to simple preparation of tissue-culture antigens of really adequate potency is illustrated by a sample calculation in terms of virus particles. Assuming the most favorable conditions imaginable in the form of a tightly packed cell monolayer nourished by fluid medium one mm. deep, on the average a square millimeter of culture surface will accommodate 1000 cells, each contributing 100 virus particles to give a maximum titer of 10^8 per ml. Since even advanced types of suspension culture cannot be expected to produce better than 10^7 cells per ml. of medium, it is apparent that complement-fixation procedures must depend on refinement of test sensitivity or concentration of viral antigens. Both methods have been used in preparation of tissue or cell culture poliovirus antigens for complement fixation. Svedmyr *et al.* (328) employed complement-fixation microtechnique in combination with concentration of tissue culture fluids by ultrafiltration. Black & Melnick (329) and Le Bouvier *et al.* (330) have used modifications of the plate complement-fixation microtest (331) with poliovirus antigens in the form of unconcentrated medium from infected monkey kidney and HeLa cell cultures. Heat inactivation of antigens was reported to reduce occasionally encountered anticomplementary activity with little effect on serologic potency. Black & Melnick concentrated insufficiently active virus preparations by ultracentrifugation. In their hands, the microtest, in which varying amounts of complement were tested with differing dilutions of serum and a constant dilution of virus, was made to yield a combined estimate of serum titer and complement-fixing capacity or avidity, as a measure of serum potency more reproducible than simple titer. Schmidt & Lennette (332) employed unconcentrated monkey kidney culture fluid without heat inactivation for poliovirus complement fixation by a macroscopic tube test. They considered this test procedure desirable for diagnostic routine application. Potency of culture-fluid antigens remained a factor limiting test sensitivity: sensitivity gained by use of larger volumes of viral antigens was offset by increased anticomplementary effects. With the plate microtest, Baumeister & Miller (333) showed that potent complement-fixing poliovirus antigens could be prepared by ultracentifugation of monkey kidney culture fluids. A satisfactory method for inactivating infectious virus without altering antigenic quality has not been found: heat treatment (334) and formalinization (335) reduced homotypic specificity of complement-fixing poliovirus preparations. The complement-fixation test with cell culture antigens undoubtedly has value both in diagnostic and epidemiological studies [Melnick *et al.* (336); Melnick *et al.* (337)] and for analysis of virus reproduction [Cohen *et al.* (291)]. While the attractive simplicity of untreated infected culture fluids as sources of complement-fixing antigens may not be realizable by virtue of insufficient specificity, it is probable that suitable antigens to produce desired results could be manufactured by chemical or physical fractionation.

Application of cell culture to virus production of vaccine has posed different and changing problems. Monolayer cultures grown from dispersed cells can be used to provide large amounts of low-protein, virus-containing fluid.

For commercial vaccine production, however, economy is a factor more important than potency; industrially, it may cost less to concentrate larger volumes of less potent virus suspension than to prepare suspensions of higher initial potency. As has been shown by the history of poliovirus vaccine production, problems of virus inactivation, preservation of antigenicity, and safety testing, may be considerably more difficult than any problems directly introduced by use of cell or tissue culture. Unless specific advantages in the form of, say, greater antigenic potency or lesser toxicity can be gained by use of true cell culture, the embryonated egg or Maitland-type tissue suspension probably will remain a preferred instrument for preparation of vaccine. When properly handled, cell culture techniques have their usual value for study of the many questions incident to vaccine development. These applications, involving qualitative or quantitative assays of infectious virus and neutralizing antibody, are exhaustively illustrated in works pertaining to the study and development of poliomyelitis and other vaccines (338 to 348). Use of cell cultures as sources of human vaccine raises three problems which deserve specific consideration. The first problem which, fortunately, did not materialize with poliovaccine, concerns the potential capacity of cell culture material on injection with or without adjuvants to sensitize vaccinees to cellular antigens or medium constituents other than virus. Failure of monkey kidney poliovaccine to induce damaging sensitization in injected humans, now well established, suggests that there might be virtually no danger from human cell cultures as vaccine sources. The second problem concerns use of malignant or potentially malignant cells as sources of material for subsequent human injection. The process of establishing cell strains may conceivably involve malignant transformation. This problem will remain beyond conclusive disproof, although any real danger from vaccine produced from malignant cells is very remote. The third problem, a practical one, was revealed in safety testing of poliovirus vaccine. While quantitative cell culture procedures can be applied effectively in the study of the kinetics and mechanics of virus inactivation, determination of safety margins by extrapolation from degradation kinetics can be dangerous, as the studies of Timm *et al.* have indicated (349). The disturbing weakness of cell culture as a vaccine safety-testing tool, suggested by findings of Eklund *et al.* (350), Gebhardt & Bachtold (351), and Syverton *et al.* (352), may be misleading. The potential value of cell culture for vaccine safety testing has not been fully investigated. For example, the membrane filters currently developed to withhold viruses might be used for safety testing. Since cells can be established and maintained satisfactorily on these filters, indicator cells could be filtered out of a trypsinized suspension to form a monolayer culture over a filter through which any desired volume of vaccine had been passed. It is possible that concomitant retention of a large amount of inactivated virus might interfere with detection of a few infectious particles.

Viral genetics, biochemistry, and immunity.—Accurate assay of intrinsic and extrinsic factors that determine response of the two protagonists of the cell-virus complex demands methods for (*a*) propagation of pure cultures of

viruses and host cells; (*b*) counting viable cells; and (*c*) counting viable virus particles. Cultures propagated *in vitro* from initially dispersed cells are suitable for truly quantitative studies of the cell-virus relation. With respect to the virus, phenomena of interest include processes of virus replication, mechanisms of viral virulence and adaptation, virus variation and recombination, and biophysical properties of intra- and extracellular virus. With respect to the cell, virus infection reflects phases of absorption, reproduction, and release. Technique for quantitative studies with cell cultures basically involves (*a*) exposure of cell monolayers or cells dispersed into suspension by action of trypsin or versene to counted numbers of virus particles, and (*b*) plaque assay of unadsorbed virus and virus contained within cells or released into supernatant fluid during or after reproduction by cellular populations or single dispersed cells. Theoretical bases for the analytic study of virus infection of cell cultures were established by Dulbecco & Vogt (55, 353) for cynomolgus monkey kidney or testis cells infected with Types 1–3 poliovirus, and for chick embryo cells infected with Western equine encephalomyelitis virus. These model studies demonstrated the single viral particle origin of plaques, the suppression of plaque formation by homotypic antiserum, the conditions necessary for isolation of clonal virus lines, the capacity of cells in monolayer culture to be infected independently, the determination of virus adsorption rates, one-step virus growth curves with cells in monolayer or suspension, the distribution of virus yields from single infected cells, and the effect of some medium constituents on virus yield. Unlike bacterial viruses, Western equine encephalomyelitis virus was seen to be released from single infected cells over long periods of time. The value of cells in suspension or monolayer as populations subject to controlled virus infection also can be exploited in comination with virus assays not involving cell culture, as shown by work of Pereira (101) with fowl plague virus, and of Rubin (252) with Rous sarcoma virus. Dulbecco & Vogt (265) later applied the analytic technique with suspensions of established-strain cells (HeLa). In collaboration with these authors, Lwoff & Lwoff (76) employed a micromanipulator together with continuous phase microscopy to study the kinetics of poliomyelitis virus release by isolated monkey kidney cells. Quantitative analysis with cell cultures yields information important practically as well as theoretically. Quantitative relations connecting latent period of infection, multiplicity of exposure, adsorption, virus yield, physical conditions of virus adsorption, and chemical conditions for virus attachment to cells [Fogh (354); Youngner (355); Levine & Sagik (356)] must be defined if accurate quantitative results from use of cell cultures are to be attained and interpreted. Genetic analysis of virus reproduction in cell cultures is somewhat hampered by the lack of a sufficient number of clear-cut virus markers. Dulbecco & Vogt (265) isolated a plaque-type mutant of poliovirus by rapid serial passage on cynomolgus kidney cells; recognition of the different plaques depended on the nutrient constitution of the agar overlay. Other plaque-type mutants of poliovirus have been described by Dubes (357). Phenomena that have been considered in the search for markers are illustrated in studies of

the biologic and serologic properties of polioviruses and their response to chemical and physical environmental factors [Melnick (295); Wenner & Kamitsuka (358); Wenner *et al.* (359); Dubes & Chapin (360); Stanley *et al.* (361)]. The technical development of cell culture to a point sufficient for definitive study of virus variation is still in progress. Advances made possible by the cell plating techniques (79) must be continued to provide a wider variety of clonal cell strains with biologic and nutritional properties more completely characterized. At present, primary interest in virus variation arises in connection with the study of virulence. Avirulent variants of polioviruses obtained by adaptive modification or other means are being sought as sources of live vaccine. Experience with propagation of polioviruses has shown that with some stains passage in tissue culture reduced virulence for monkeys or mice [Melnick (362); Li & Schaeffer (363)], sometimes abruptly. It is not clear what mechanism was responsible for this host-controlled variation; the problem may be difficult to resolve until virulence of virus for animals is related to capacity to propagate in particular cells *in vivo*. While it is difficult to investigate *in vivo* trophism and propagation of virus, the use of cell culture to approach the problem is illustrated in the work of Li & Jahnes (364). Mouse-adapted Leon strain, Type 3 poliovirus, abruptly lost mouse virulence after a brief passage in monkey testis but retained some virulence for mice after such passage in monkey kidney culture, although comparable tissue culture titers were attained in both types of cultures. A single passage in monkey testis or kidney tissue grafted on chick embryo chorioallantoic membrane restored the mouse virulence. although tissue culture titer again was unaltered. In contrast, another poliovirus strain was said to have gained mouse virulence by passage in monkey testis culture. The probability of modifying virulence by cultivation of virus *in vitro* seems to increase with rapid serial passage of large virus inocula [Sabin *et al.* (365)]. Studies of virulence modification and associated properties of poliovirus are being continued [Stanley *et al.* (366, 367, 368)]. Another cell-virus system useful for the study of virulence modification was investigated by Moore & Diamond (369). Adsorption of Newcastle disease virus by mouse ascites cells inhibited tumor growth *in vivo;* adsorption at 37°C. resulted in irreversible inhibition; viral multiplication was not demonstrable *in vitro* or *in vivo*. Propagation in cell culture, as shown by egg titration, later was found to be dependent on the use of adequate culture medium and transfer of cells rather than on culture fluid. After adaptation to tumor-cell culture, virus appeared in fluid as well as in cells. Evidence of virus growth in tumor-cell cultures also was shown by titrations of culture fluid with minced chick embryo tissue cultures. Continued incubation of virus with tumor cells resulted in progressively reduced viability of the tumor cells by test *in vivo*. Virus adapted to propagation in tumor-cell culture consistently failed to grow in ascites mouse tumor cells *in vivo*. An unusual opportunity to investigate the effect of adaptive modification of virulence on other virus characters resulted from similarity between viral hemagglutination and the virus adsorption here reflected by inhibition of tumor-cell growth. By comparison

with parental stocks, elution of culture virus from tumor cells occurred less easily,—either spontaneously or by the influence of antiserum, receptor-destroying enzyme, or washing. The contributions of cell culture technique to virus genetics are presently concerned more with exploration of the extent of virus variation than with the influence of mechanisms of selection, induction, or transformation (370). The usefulness of such possible virus markers as serologic type, cellular range of infectivity, cellular yield of virus, and radiation sensitivity [Youngner *et al.* (371); Youngner (372); and Fogh (373)] has been increased by simplification of monolayer plaque technique (56). Experiments designed to detect viral recombination [van den Ende & Selzer (374); Sprunt *et al.* (375)] demonstrate the greater difficulty attending use of tube culture dilution techniques rather than plaque assays for identification of virus variants.

Geneticists, biochemists, and cellular physiologists have a common interest in the advancement of cell culture procedures: the ultimate hope is to explain virus reproduction in terms of enzymes, metabolites, and DNA molecules. Since these are synthesized by the cell, development of virus biochemistry represents a contribution to cell biology as well. The *in vitro* culture system, by elimination of complex secondary responses of an intact host to virus infection, has simplified investigation of the cell-virus relationship; the use of cells rather than tissue cultures satisfies additional requirements for (*a*) reproducibility of results, by use of replicate cultures of homogeneous constitution; (*b*) quantitation of reagents, by determination of numbers of infectious virus particles and numbers or mass of cells; and (*c*) increased control of physical and chemical environment, by elimination of plasma coagulum and the uncontrollable intercellular relations of mixed cell populations. In the absence of greater knowledge of the physiology of uninfected cells, research on virus biochemistry has been largely restricted to comparison of normal and infected cells by the usual methods. These include (*a*) assay of essential nutritional elements; (*b*) alteration of chemical and physical environments; (*c*) application of antimetabolites; and (*d*) assay of enzymes. Current development of new biochemical micromethods, and of mass cultivation of animal cells in suspension to provide materials sufficient for conventional biochemical analysis, is facilitating research. Many aspects of virus biochemistry related and unrelated to animal cell culture have been reviewed [Pearson (376); Cohen (377); Matthews & Smith (378); Hurst & Hull (379); Putman (380)]. Here, application of cell culture to biochemical study is emphasized rather than results, and references have been selected accordingly. The biochemist should understand that biochemical analyses cannot be interpreted meaningfully except on the basis of proper appreciation of the biology of cells and virus infection. Dulbecco (258) has considered some of the assumptions, definitions, and principles pertinent to the integrated study of several animal cell-virus interactions. Definition of essential nutrients for virus reproduction is more complicated with animal cells than with bacteria, partly because nutrient media are complex. The demonstra-

tion that virus propagation could be accomplished by a variety of tissues maintained in synthetic medium (30, 381, 382, 383) was helpful in showing that a complex medium essential for cell growth was not necessary for virus replication. Experiments with simplified culture systems have shown that surprisingly few nutrient materials are required by cells for virus infection and replication: for instance, monkey kidney cells maintained in Eagle's medium without serum exhibited retarded cytopathic effect and delayed propagation of Types 1–3 poliovirus only when cystine, among the vitamins and other amino acids ordinarily incorporated in the medium, was omitted [Dubes (384)]. In devising a simple synthetic medium for the preparation of monkey kidney cultures, Rappaport (38) had found cysteine the only nutritional supplement necessary for synthesis of Type 1 poliovirus. While such findings are practically useful, the need for caution in fundamental interpretation is made clear by the experiments of Eagle & Habel (385). HeLa cells maintained in a glucose-glutamine-salts medium produced only about one log less than the maximum yield of virus despite 12 hr. deprivation of other nutrients found in complete medium. Even this reduction in yield may have been a consequence of accelerated culture degeneration since maximum yield was restored when cellular degeneration was somewhat delayed by the addition of from three to five per cent dialyzed serum to the simple medium. Indeed, omission even of glucose and glutamine appeared to affect duration rather than rate of virus production by the HeLa cultures. The immense decrease in net viral yield resulting from the omission of glucose and glutamine from either simple or complete medium, indicated that only a fraction of the culture cells was contibuting virus; it was not known whether the virus yield per cell also was decreased. Animal cells, to a much greater extent than bacteria, appear able to synthesize virus almost completely from material provided by their own nutrient reserve. Eagle & Habel have pointed out that nutritional components demonstrably essential may act only to preserve cellular integrity long enough for virus synthesis. It is apparent that the most advanced cell culture techniques, so applied as to yield results showing both rate and amount of virus yield on a per cell basis, are necessary to fundamental research on animal virus biochemistry. Furthermore, investigators not uncommonly fail to define which growth factors or other nutrients are contributed to a supposedly defined medium by cells undergoing destruction in a cell population maintained by the defined medium under something less than physiologically optimum conditions. Active phagocytosis of cellular debris appears to be common to all cells in primary or continuous culture. It is essential to know the extent of cell damage or destruction during the process of cell culture and production of virus, for adequate interpretation of the capacity of a given medium to support virus synthesis. Processes which operate for cellular maintenance and virus replication are almost inseparable. Concomitant assay of cellular metabolism, then, seems essential to the nutritional study of virus infection; greater use of antimetabolites and radioactive tracers, as indicated by past and present research, should

aid solution of the problem. Additions to the present number of established cell strains of clonal origin, and of viruses propagable in cultures of such cells, also may facilitate research by providing a variety of synthetic systems differing in complexity. Requirements of some cells for virus synthesis may not be as simple as those of HeLa and monkey kidney cells: Scherer (34), for example, found that strain L cells maintained in serum ultrafiltrate medium produced much less herpes simplex or pseudorabies virus than cells proliferating in serum medium supplemented with embryonic extract. Similarly, Morgan and associates (386, 387, 388) found that multiplication of psittacosis virus in infected chick embryo cells could be delayed by maintenance of the cells in a glucose-salts medium; virus replication was reactivated at will by supplementation of the simple medium with natural or defined nutrients including phenylalanine and tryptophan, among other amino acids and vitamins. The supply of nutrient or specific composition of the medium can affect cytopathogenic response of virus-infected cells [Reissig *et al.* (389)] as well as their capacity to grow virus [Vieuchange (390)]. The use of net virus yield by infected cell cultures to measure the effects on virus synthesis depends on careful definition of physical and chemical conditions affecting virus stability [Ginsberg (391)] since, with unstable virus, the final assayed yield may reflect equilibrium between virus production and inactivation. The effects of physical and chemical environments on rates of cellular physiological activity also must be taken into account, since these rates will, in turn, affect rate and duration of virus production and hence ultimate virus yield. The quantitation possible with cell strains propagable from dispersed cell suspensions makes them superior to explant or other mixed tissue or cell cultures for evaluation of environmental influences (89); primary cultures are nonetheless useful for other purposes, such as evaluation of the possible role of environmental influences in differential virus susceptibility of tissues *in vivo* [Colville *et al.* (392)]. The value of an experimental method involving concomitant and independent measurement of virus yield and metabolic activity of host cells is illustrated by the findings of Gifford *et al.* (105) which relate influence of medium pH on replication of Type 1 poliovirus by HeLa cells to the effects on cellular metabolism. In all physiological studies of virus production by strains of cells in continuous culture, it is essential that controls be tested at the same time in the same laboratory; designation by the same label of cell strains maintained under varying conditions in different locations is no guide to similarity of their physiological responses [Scherer (393)]. Furthermore, if studies of the influence of environmental alterations or metabolite analogues on virus production *in vitro* [Cushing & Morgan (394); Eaton & Scala (395); Levine *et al.* (396)] are to be related to phenomena *in vivo*, all factors affecting behavior of cell cultures must be appreciated and evaluated. Robertson *et al.* (397) observed that heated ribose inhibited poliovirus synthesis by HeLa cells, implicated furfural as the inhibitory factor, and found that furfural protected cells in stationary culture against poliovirus infection. Furfural was inactive in agitated cultures and in orally infected

monkeys. In considering these effects, it was necessary to distinguish between any intrinsic action of furfural on virus infection and its effect on virus or cells: some of the protection conferred on stationary cultures resulted indirectly from slowed cellular metabolism consequent to increased acid production. Without detailed knowledge of metabolic pathways operative in uninfected cells in culture, it is difficult to assess changes in metabolic or enzymic activity produced by virus infection. At present, it is necessary to explore the effects of virus infection on cellular metabolism before mechanisms can be defined. For such a study, cell culture technique makes available cell populations which are uniform in physiological type and susceptibility to virus, compared to explant cultures previously used, and which permit nearly simultaneous infection of most population members. Problems of quantitation are reduced but not eliminated by use of cell cultures; it may be useful, as shown by Levy & Baron (398) to work with purified virus in order to eliminate the influence of nonviral components of inoculum on changes occurring shortly after infection of cells. In studying changes in enzyme activity of infected cells [Kovacs (399)], means must be found for relating enzyme activity to viable cell mass in order to distinguish effects on enzyme concentration brought about by destruction of cells in infected cultures. The use of cell cultures for study of virus infection is interesting from the viewpoint of cell physiology as well as virology, since effects of physiological agents such as hormones [Eaton *et al.* (400)] on cellular synthetic capacity represented by virus production can be conveniently assayed.

An aspect of cell-virus relationships deserving further investigation is the specific immunity of cells to viruses in the absence of protective mechanisms common to the intact animal. Such immunity could be based on: (*a*) lack of suitable cell receptors so that virus adsorption is prevented; (*b*) failure of cells to reproduce absorbed virus in infectious form; (*c*) capacity of cells to harbor latent virus analogous to temperate bacteriophage with or without immunity to superinfection; and (*d*) interference between viruses. While mixed-cell cultures have been employed successfully for the demonstration of interference *in vitro* [Chanock (240); van den Ende & Selzer (374); Taylor (401); Ledinko & Melnick (402); Sabin (403); Le Bouvier (404); Ivânovics *et al.* (405)] the full advantages of clonal cell strains are needed for the investigation of mechanisms of this and other examples of cellular immunity to viruses. As Ackermann & Kurtz (406) point out in their discussion of a persisting poliovirus infection of HeLa cells continually propagated in immune-serum medium, it is necessary to differentiate between the apparent immunity of whole cultures and the immunity of individual cells. A further example is provided by the work of Kaplan & Melnick (407) which shows that propagation of infectious Type 1 poliovirus by capuchin monkey kidney cultures without apparent cell destruction resulted from virus reproduction by a visually undetectable fraction of the cell population. A method for analysis of some cellular immunity phenomena has been indicated by Puck & Marcus (155); HeLa cells apparently harboring latent virus were said to

produce large plaques when plated on monolayers of giant HeLa cells. These giant cells, resulting from x-irradiation of HeLa cultures, apparently were more sensitive to virus infection. It is not known whether susceptibility of all cells can be similarly increased. Schneider & Cheever (408) observed similar rates of vaccinia virus propagation in control and irradiated chick embryo tissue cultures. Cultivation of cells permits comparison of the viral susceptibility of cells in and outside the intact animal [Evans *et al.* (195, 409)], for characterization of cellular immunity *in vivo*. By propagating cells dispersed from tissue, location and multiplicity of exposure to virus *in vivo* can be determined quantitatively. In experiments by Kaplan (410), kidneys of living susceptible monkeys were inoculated with poliovirus, removed at varying times, and homogenized for assay of the contained virus or trypsinized for preparation of cell cultures. Virus content of kidney cells kept within the monkey declined considerably while that of cells removed to culture *in vitro* increased manyfold. Aliquots of kidney cell suspension were homogenized and assayed for virus, or plated on monolayers of susceptible cells for assay of infective centers; similar ratios of virus/cell concentrations were found, suggesting adsorption of virus *in vivo* without replication. Acquired tissue immunity to virus infection likewise can be studied with tissue or cell culture. Pollard (411) found that explant cultures of spleens taken from mice immunized with St. Louis encephalomyelitis or mouse encephalomyocarditis viruses were selectively immune to homologous virus and susceptible to heterologous virus. Assays were not done which would reveal any influence of antibody produced *in vitro*.

Summary

To summarize, the contributions of cell culture to virus research in the last six years include provision of (*a*) a varity of animal cells in stable continuous culture, propagable from dispersed suspensions; (*b*) improved and simplified chemical or enzymatic means for dispersing cells from tissue or from monolayer cultures; (*c*) growth and maintenance media of largely defined composition; (*d*) methods for derivation of cell lines of clonal origin; and (*e*) culture methods for short- or long-term maintenance of cells in suspension, for plaque assay of infectious virus particles, and for analysis of virus infection in single cells. This period of virus research with cell cultures has been dominated by the practical goals of poliovirus study and its influence on the investigation of other viruses. Research mostly has been concerned with the elaboration of relatively simple qualitative procedures for large-scale use of tissue cells in primary culture for diagnostic and epidemiological use, with expansion of the list of stock stable strains of cells and characterization of their viral susceptibility and range of cytopathogenic response, and with improvement and refinement of cell culture methodology. It may be anticipated that the coming period will see continued application of present cell culture methods to diagnostic and epidemiologic studies of a wider variety of viruses including many which are responsible for specific diseases, others now known as orphans, and viruses still undiscovered. The need for

antiviral chemotherapeutic agents undoubtedly will continue to stimulate fundamental research on virus infection and reproduction in animal cells and on cellular physiology. If the potential value of cell culture techniques for virus research is to be realized fully, work must be continued on (*a*) search for wholly adequate and completely defined media for cultivation of cells; (*b*) characterization of metabolic pathways normally operative in cultivated cells; and (*c*) on a definition of virus markers and evolution of indicator cell lines so that the study of animal-virus genetics can be pursued with the powerful techniques proven to be so fruitful with bacterial viruses.

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